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**Proteomic Profiling of an Opportunistic Human and  
Animal Pathogen *Staphylococcus epidermidis***

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University of Helsinki  
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**Proteomic profiling of  
an opportunistic human and animal pathogen  
*Staphylococcus epidermidis***

**Pia Siljamäki**

ACADEMIC DISSERTATION

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*“Learn from yesterday,  
live for today,  
hope for tomorrow.  
The important thing is  
to not stop questioning.”  
- Albert Einstein*

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## LIST OF ORIGINAL PAPERS

This thesis is based on the following original articles that are referred to in the text by their Roman numerals I-III.

- I Kirsi Savijoki, Antti Iivanainen\*, Pia Siljamäki\*, Pia K. Laine, Lars Paulin, Taru Karonen, Satu Pyörälä, Matti Kankainen, Tuula A. Nyman, Tiina Salomäki, Patrik Koskinen, Liisa Holm, Heli Simojoki, Suvi Taponen, Antti Sukura, Nisse Kalkkinen, Petri Auvinen, Pekka Varmanen (2014). Genomics and Proteomics Provide New Insight into the Commensal and Pathogenic Lifestyles of Bovine and Human-associated *Staphylococcus epidermidis* strains. *J. Proteome Res.* 2014 Jul 11. doi: 10.1021/pr500322d. \* = equal contribution
- II Pia Siljamäki, Pekka Varmanen, Matti Kankainen, Satu Pyörälä, Taru Karonen, Antti Iivanainen, Petri Auvinen, Lars Paulin, Pia K. Laine, Suvi Taponen, Heli Simojoki, Antti Sukura, Tuula A. Nyman, Kirsi Savijoki (2014). Comparative Proteome Profiling of Bovine and Human *Staphylococcus epidermidis* Strains for Screening Specifically Expressed Virulence and Adaptation Proteins. *Proteomics*, 2014 Jun 7. doi: 10.1002/pmic.201300275.
- III Pia Siljamäki, Pekka Varmanen, Matti Kankainen, Antti Sukura, Kirsi Savijoki, Tuula A. Nyman (2014). Comparative Exoprotein Profiling of Different *Staphylococcus epidermidis* strains Reveals Potential Link between Non-classical Protein Export and Virulence. *J Proteome Res.*, 2014 Jun 2. doi: 10.1021/pr500075j.

## ABBREVIATIONS

CoNS = coagulase-negative *staphylococci*

COG = clusters of orthologous groups

ESI = electrospray ionization

GeLC-MS/MS = one-dimensional gel electrophoresis coupled with liquid chromatography – tandem mass spectrometry

GO = gene ontology

IEF = isoelectric focusing

IMI = intramammary infection

IMP = integral membrane protein

LC-MS/MS = liquid chromatography – tandem mass spectrometry

MALDI-TOF MS = matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry

MSCRAMM = microbial surface components recognizing adhesive matrix molecules

MRSA = methicillin-resistant *Staphylococcus aureus*

MRSE = methicillin-resistant *Staphylococcus epidermidis*

MV = membrane vesicle

OMV = outer membrane vesicle

ORF = open reading frame

PGA = poly- $\gamma$ -glutamic acid

PIA = polysaccharide intracellular adhesion

PSM = phenol soluble modulin

SA = *Staphylococcus aureus*

SCV = small colony variant

SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis

SE = *Staphylococcus epidermidis*

TAT = twin arginine pathway

TCA = tricarboxylic acid

TMD = transmembrane domain

TOF = time-of-flight

2-DE = two-dimensional gel electrophoresis

2D DIGE = two-dimensional difference gel electrophoresis



## ABSTRACT

*Staphylococcus epidermidis* (SE) is an opportunistic pathogen capable of infecting humans and animals. It is a frequent cause of hospital-acquired infections in humans and intramammary infections (IMIs) in dairy cows. The aim of this study was to compare the protein expression profiles and the genomes of three SE strains, one associated with bovine mastitis (PM221), one representing a commensal/low-virulent human strain (ATCC12228 isolated from a healthy human host) and the third being a virulent human strain (RP62A isolated from a catheter-associated sepsis), in order to reveal the factors employed by this species for virulence, survival and adaptation.

The genome-level analysis (I) revealed that the genome of a SE strain PM221 isolated from subclinical bovine IMI resembled more the commensal-type, low-virulent human strain ATCC12228 than the virulent human strain RP62A. While a comparative proteome analysis and the total proteome profiling (I, II) confirmed the close relationship between the bovine PM221 and the commensal-type ATCC12228, certain strain-specific differences were found which are believed to have roles in adaptation and virulence. These findings may explain why ATCC12228 was able to cause persistent mastitis in an experimental infection study but with a milder clinical outcome than encountered with the bovine strain PM221. Taken together, these findings strengthen the hypothesis that humans could represent an important source of SE-mediated infections in dairy cows.

The exoproteomes of the three SE strains were characterized (III) in an attempt to investigate the infectious potential in more details. This study showed that the PM221 and RP62A exoproteomes were more similar, differing from that of ATCC12228. The major part of the identified exoproteins were predicted to be cytoplasmic, indicating that these proteins might be surface-displayed moonlighting proteins released into the culture medium via some yet uncharacterized mechanism, or that they had been embedded in membrane vesicles (MVs) that export proteins in a concentrated and protected manner. The results also suggest that these non-classical protein secretion pathways were being more efficiently exploited by PM221 and RP62A. In summary, the exoproteomic results can explain the higher virulence capacity of PM221 compared to ATCC12228.

Phenotypic tests were conducted to confirm some of the major proteomic results. These tests indicated that SE may use several strategies to improve its adaptation to different environments; the virulent bovine and human strains (PM221 and RP62A) were able to use biofilm formation for colonization and adaptation, and PM221 and the commensal-type human strain (ATCC12228) could downregulate their tricarboxylic acid cycle activity and increase the formation of small colony variants to improve bacterial survival during the stationary phase. In addition, the bovine PM221 strain may possess an advantage since it has higher tributyrin activity in the milk environment, whereas the human strains may benefit from their higher urease and/or catalase activities helping these strains to survive in their ecological niches (i.e. on human skin, mucous membranes) and indwelling medical devices.

In conclusion, the present studies demonstrate that the SE species may exploit diverse strategies involving specific changes in their late stationary phase metabolism as well as in protein export strategies accompanied by increased production of certain enzymes in order to ensure better adaptation and/or successful infection.

# 1 INTRODUCTION

## 1.1. SE is an opportunistic human and animal pathogen

The Gram-positive bacterium *Staphylococcus epidermidis* (*SE*) is a coagulase-negative staphylococcus (CoNS) that is a normal habitant of human skin and mucous membranes. As a part of human microbiota, it has a benign relationship with the host, and it is even able to hinder the colonization of potentially more harmful microbes, such as *Staphylococcus aureus* (*SA*), thus it can be considered as a “skin probiotic” conferring protective immunity. (Cogen, Nizet et al. 2008, Iwase, Uehara et al. 2010, Wanke, Steffen et al. 2011, Al-Mahrous, Jack et al. 2011, Naik, Bouladoux et al. 2012, Sugimoto, Iwamoto et al. 2013)

Despite the fact that *SE* is the most frequently encountered CoNS species on human skin and that it normally adopts a commensal lifestyle, it is also by far the most frequent source of infections caused by CoNS in humans (Otto 2009). Since *SE* is also able to infect animals (Pyörälä, Taponen 2009), it can be viewed as a pathogen with both medical and veterinary relevance. For example, it has been estimated that at least 22% of the bloodstream infections associated with central vascular catheters and about 13% of the prosthetic valve endocarditis infections are caused by *SE* (Otto 2009). CoNS are the predominant cause of bovine intramammary infections (IMIs) in several countries, and *SE* is one of the five CoNS species (*Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus haemolyticus*, *Staphylococcus xylosus*, and *Staphylococcus epidermidis*) most frequently identified as IMI causing species (Thorberg, Danielsson-Tham et al. 2009, Moser, Stephan et al. 2013, Vanderhaeghen, Piepers et al. 2014).

### 1.1.1 SE as a nosocomial human pathogen

*SE* is a major causative agent of hospital-acquired, nosocomial infections. *SE* infections are typically associated with indwelling medical devices such as heart valves, catheters and optical lenses especially in immunocompromised patients, newborns and elderly individuals. (von Eiff, Peters et al. 2002, Otto 2009, Rogers, Fey et al. 2009, Schoenfelder, Lange et al. 2010, Otto 2012) These infections often cause only moderate clinical symptoms, and are seldom life-threatening, because *SE* does not produce many aggressive virulence factors, such as toxins or exoenzymes, or produces them only in moderate amounts (Zhang, Ren et al. 2003, Gill, Fouts et al. 2005, Otto 2012). Nevertheless, the medical implications of nosocomial *SE* infections are substantial, as the treatment is usually difficult, and the infections tend to become chronic.

One of the reasons behind the rise of *SE* as a nosocomial pathogen is the extensive and expanding use of biomaterials in medicine, such as catheters, heart valve prostheses, orthopedic implants and pacemakers (Campoccia, Montanaro et al. 2013). These devices are prone to *SE* colonization and infection because they offer suitable surfaces onto which *SE* can adhere and colonize. Once attached onto a surface, *SE* is able to form biofilms, which are surface-attached bacterial communities embedded in a “sticky” extracellular matrix (Costerton, Stewart et al. 1999). Once this slimy bacterial biofilm has been formed, the treatment becomes difficult as the microbial cells within the biofilm are well protected. An additional challenge is due to the fact, that *SE* strains are also frequently resistant to many antibiotics. (Costerton, Stewart et al. 1999, Gill, Fouts et al. 2005, Otto 2012, Otto 2013b, Begovic, Jovicic et al. 2013) The lack of response to medical treatments leads to persistent and chronic infections requiring removal of the device, which in turn may mean extra surgical operations and longer hospital stays. In addition, biofilms repeatedly release bacteria into the bloodstream and other tissues, causing

severe secondary infections, such as bacteremia, endocarditis and osteomyelitis, which in turn significantly increase morbidity and mortality. (Huebner, Goldmann 1999, Mack, Davies et al. 2007, Van Mellaert, Shahrooei et al. 2012, Fey, Olson 2010) Yet another risk associated with *SE* in hospital environments originates from the fact that *SE* can serve as a reservoir of antibiotic resistance genes to be horizontally transferred to *SA*, leading to the emergence of the notorious methicillin-resistant *SA* (MRSA) (Wielders, Vriens et al. 2001, Bloemendaal, Brouwer et al. 2010, Otto 2013a).

As the human population is aging, the use of indwelling medical devices increases. At the same time, the wide-scale use of antibiotics and disinfectants is creating a favorable environment for the rise of resistant and well-adapted strains in hospitals. For these reasons, the number of difficult nosocomial infections caused by *SE* has been predicted to continue to increase in the future (Schoenfelder, Lange et al. 2010, Van Mellaert, Shahrooei et al. 2012).

### **1.1.2 *SE as a mastitis causing animal pathogen***

In addition to being a human pathogen, *SE* is also one of the major causative species of intramammary infections (IMI) in livestock such as dairy cattle (Taponen, Simojoki et al. 2006, Taponen, Koort et al. 2007, Mørk, Jørgensen et al. 2012, Taponen, Pyörälä 2009, Fitzgerald 2012, Pyörälä, Taponen 2009, Thorberg, Danielsson-Tham et al. 2009, Moser, Stephan et al. 2013, Vanderhaeghen, Piepers et al. 2014). Like human *SE* infections, also *SE* IMIs are usually only mildly clinical or even subclinical, but often persistent (Taponen, Simojoki et al. 2006, Taponen, Koort et al. 2007, Taponen, Pyörälä 2009, Thorberg, Danielsson-Tham et al. 2009, Zadoks, Watts 2009, Mørk, Jørgensen et al. 2012). Unlike the situation in humans, *SE* is not a part of the normal microbiota of bovine skin or mucous membranes (Thorberg, Kuhn et al. 2006, Jaglic, Michu et al. 2010). The *SE* strains that have been isolated from bovine IMI share the same genotype with those strains that have been isolated from the milker's skin. They also seem to produce the same virulence factors and antimicrobial resistance patterns as the strains that are isolated from human infections (Thorberg, Kuhn et al. 2006, Sawant, Gillespie et al. 2009, Jaglic, Michu et al. 2010). These observations have led to the hypothesis that cows may acquire *SEs* from the humans that are working in the barn environment, i.e. humans could be the source of mastitis causing *SEs* (Thorberg, Kuhn et al. 2006, Jaglic, Michu et al. 2010, Piessens, Van Coillie et al. 2011).

The frequent occurrence of IMI in dairy herd results in animal suffering, in extensive use of antibiotics, and in significant financial losses due to the veterinary costs and reductions in milk production. The large-scale use of antibiotics also raises concerns about the spread of antibiotic resistance and food safety (Boehmer 2011, Walther, Perreten 2007). This concern for antibiotic resistance is even more pronounced in *SE* than in other mastitis causing CoNSs, as the milk-isolated *SE* strains exhibit greater phenotypic resistance to multiple antimicrobials than the other milk-isolated CoNSs (Sawant, Gillespie et al. 2009).

## **1.2 The molecular basis of *SE* infections**

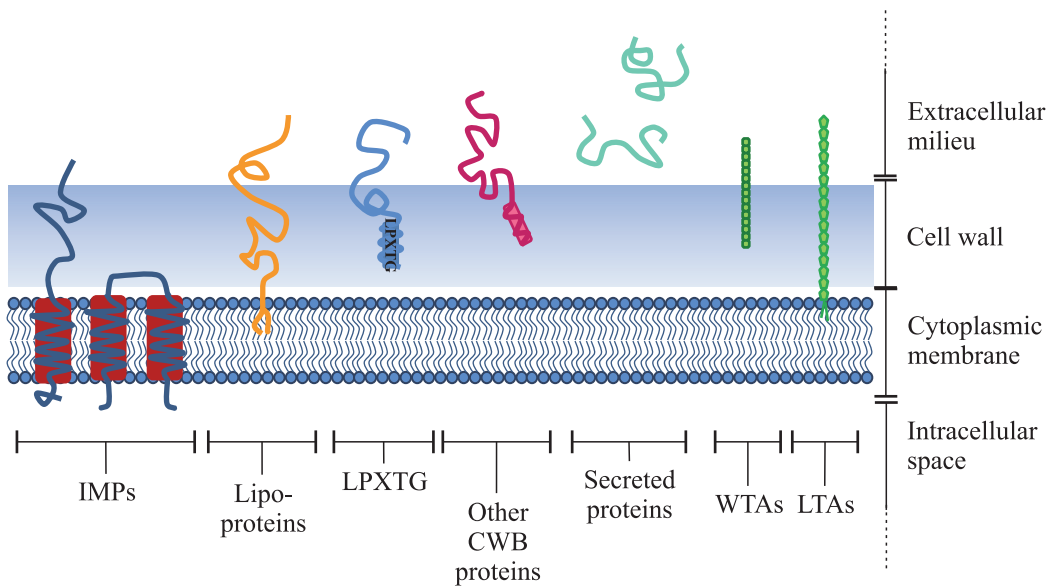
The central aspects of *SE* pathogenicity are the molecules and mechanisms that these bacteria use to interact with the surrounding environment, and its ability to survive in the host. The staphylococcal virulence factors have been broadly categorized into four factors i.e. those that are i) involved in adhesion and invasion, ii) mediating degradation of host cells, iii) enabling the bacteria to evade host immune response, and iv) involved in the utilization of host-derived nutrients (Hecker, Becher et al. 2010).

Proteins are important for the interaction with the environment. In order to interact with the environment, these proteins must be exposed outside the bacterium either as attached to the cell wall and/or cell membrane (i.e. surfacome) or secreted out of the cell (i.e. exoproteome) as freely soluble proteins or encapsulated in vesicle structures. These proteins have good opportunities to interact with the environment, and to be recognized by the host immune system and thus to act as antigens or immunomodulators. Therefore they are often regarded as targets for new vaccines and diagnostic tools and in this respect they are of major medical importance. (Dreisbach, van Dijl et al. 2011, Olaya-Abril, Jimenez-Munguia et al. 2013) The virulence factor that is regarded as the single most important contributor in SE's survival and pathogenicity in humans is its ability to form and grow as a biofilm, a phenomenon that is also dependent on exoproteins. (Fey, Olson 2010, Otto 2012, Otto 2013b)

### **1.2.1 Cell wall/membrane-associated surface proteins and secreted proteins**

The cell envelope that surrounds the bacterial cell serves many vital functions. For example, it maintains cation homeostasis, exports and imports various important ions and molecules, and serves as a platform for surface proteins (Scott, Barnett 2006). The cell envelope of Gram-positive bacteria consists of a cytoplasmic membrane and an outer cell wall. The cytoplasmic membrane is a phospholipid bilayer that contains integral membrane proteins (IMPs). The cell wall that surrounds the cytoplasmic membrane is a cross-linked peptidoglycan mesh containing anionic polymers called teichoic acids threading through the peptidoglycan layers, plus a wide variety of surface-attached proteins and other polymers (Figure 1). (Desvaux, Dumas et al. 2006, Scott, Barnett 2006, Silhavy, Kahne et al. 2010)

The bacterial surface proteins are expressed extensively during the exponential growth phase. For example, they protect the bacteria from environmental hazards, promote nutrition acquisition, attach to extracellular components, undergo interactions with other bacteria and the host cells, carry out enzymatic activities and help with immune evasion (Scott, Barnett 2006, Olaya-Abril, Jimenez-Munguia et al. 2013). The four major types of cell surface proteins in Gram-positive bacteria are i) integral membrane proteins (IMPs) passing the cell membrane with trans-membrane domains, ii) lipoproteins that are covalently attached to the membrane lipids via an N-terminal lipobox that mediates covalent binding of a conserved Cys residue to a lipid, iii) cell-wall associated proteins that are covalently attached to the peptidoglycan wall through the LPXTG-anchoring motif, and iv) other proteins that bind to distinct cell wall components with specific binding domains (Figure 1). These specific binding domains (iv) include leucine-rich repeat region (LRR) that is involved in protein-protein and protein-ligand interactions; LysM is a C-terminal lysine mediating non-covalent attachment to peptidoglycan; C-terminal anchoring signal motifs LPXTG, NPQTN and EVPTG for sortase-mediated protein anchoring; and TLXTC, a sortase signature motif with a catalytic cysteine residue. (Mazmanian, Ton-That et al. 2002, Desvaux, Dumas et al. 2006, Scott, Zahner 2006, Scott, Barnett 2006, Matsushima, Miyashita et al. 2010).



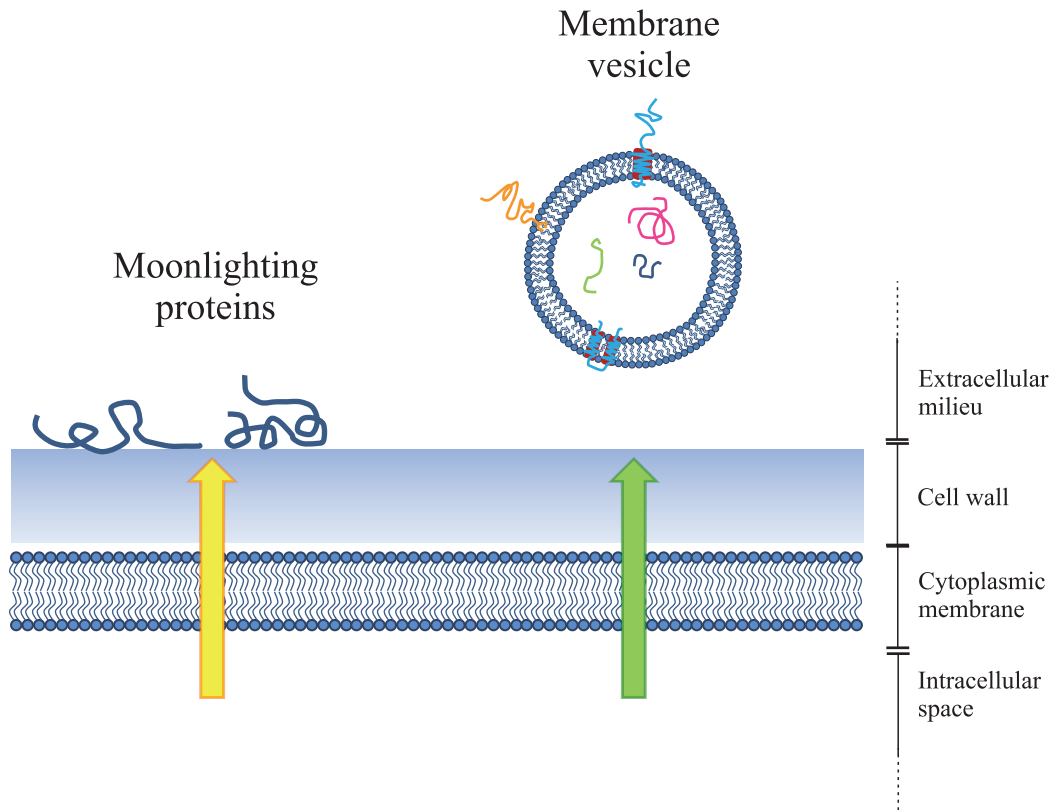
**Figure 1.** Major types of cell surface proteins in Gram-positive bacteria: IMPs (integral membrane proteins) with trans-membrane domains; lipoproteins attached to the lipids via the lipobox; proteins that covalently attach to the cell wall through LPXTG-anchor; and other cell wall-attached proteins (CWBs) with distinct anchoring motifs (LysM, a C-terminal lysine mediating a non-covalent attachment to peptidoglycan; C-terminal sorting signal motifs EVPTG, NPQTN and TLXTC; LRR, a leucine-rich repeat region involved in protein-ligand and protein-protein interactions). The figure shows also proteins secreted out of the cell, and wall teichoic acids (WTAs) / lipoteichoic acids (LTAs) spanning the cell wall and attaching to peptidoglycan (WTAs) and membrane lipids (LTAs).

At the time when the bacterium enters into the late exponential growth phase, the expression and export of proteins shifts from surface proteins towards those that are secreted into the extracellular milieu (Figure 1). These secreted proteins also serve vital tasks in bacterial survival, communication between cells, response to environmental stress, and nutrient acquisition (Tjalsma, Antelmann et al. 2004), and they include many virulence factors, such as proteases, lipases, nucleases and autolysins. (Bowden, Chen et al. 2005, Otto 2012) For example, the secreted proteases contribute to bacterial virulence by scavenging nutrients (Dubin 2002), while secreted lipases are regarded as important players in the colonization of lipid-rich environments such as the skin, the main living niche for *SE* (Simons, van Kampen et al. 1998, Bowden, Visai et al. 2002, Powers, Smith et al. 2011).

### 1.2.2 Non-classical secretion - moonlighting proteins and membrane vesicles

Gram-positive bacteria use several mechanisms to secrete proteins out of the cell. The so-called classical protein secretion mechanisms include for example the general secretory (Sec) pathway and the twin arginine (TAT) pathway. The most common export system is the Sec system that requires the presence of an N-terminal signal sequence in the protein to be secreted. (Scott, Barnett 2006, Powers, Smith et al. 2011, Freudl 2013) While the classical secretion mechanisms have been widely studied in Gram-positive bacteria (Scott, Barnett 2006, Powers, Smith et al.

2011, Freudl 2013), these are not the only ways with which the bacterium can export proteins into the extracellular milieu. There are also non-classical and much less studied secretion systems e.g. moonlighting proteins and membrane vesicles (MVs) (Figure 2) (Kainulainen, Korhonen 2014, Lee, Choi et al. 2009).



**Figure 2.** Non-classical secretion systems in SE. Left: Moonlighting proteins attached to the cell surface. Right: Protein-containing secreted membrane vesicle. (Not in scale)

Moonlighting is a rather recently discovered aspect of bacterial exoproteome of both pathogenic and commensal bacteria. Proteins are said to be moonlighting when they express more than one function and these functions take place at different cellular locations (Henderson, Martin 2011). The canonical functions of these proteins are involved in central cellular functions, such as glycolysis, chaperone activity, protein synthesis, and stress responses, and occur in the cytoplasm. Their moonlighting functions have often been associated with adhesion to extracellular components thus contributing to host-microbe interactions, and these take place on the cell surface. (Pancholi, Chhatwal 2003, Carneiro, Postol et al. 2004, Xolalpa, Vallecillo et al. 2007, Henderson, Martin 2011, Wang, Xia et al. 2013, Kainulainen, Korhonen 2014) Some moonlighting proteins can also act as receptors for e.g. the complement system subunits and modulate host immune responses. (Agarwal, Hammerschmidt et al. 2012, Wang, Kelly et al. 2001, Terao, Yamaguchi et al. 2006, Bhattacharya, Ploplis et al. 2012, Henderson, Martin 2011). In commensal bacteria, moonlighting proteins have similar functions as in pathogenic bacteria, such as adhesion (Antikainen, Kuparinen et al. 2007a, Kinoshita, Uchida et al. 2008,



Kainulainen, Loimaranta et al. 2012), possibly helping the commensal bacteria to compete for receptors against pathogens and therefore they occupy the attachment sites preventing binding of pathogenic species (Spurbeck, Arvidson 2010).

It is not yet known how these moonlighting proteins are secreted outside of bacterial cells. They might be exported through some yet unknown secretion mechanism or – as they do not seem to have any known secretion or anchoring signals – they may be released from traumatized and/or lysed cells (Kainulainen, Korhonen 2014). These two alternatives are not mutually exclusive, and there has been evidence supporting both hypothesis (Boel, Pichereau et al. 2004, Boel, Jin et al. 2005, Yang, Ewis et al. 2011) as well as for the hypothesis that they are released as a part of a cellular stress response (Eichenbaum, Green et al. 1996, Candela, Centanni et al. 2010, Kainulainen, Loimaranta et al. 2012). It has been suggested that the moonlighting proteins use ionic interactions to attach themselves to the cell surface, for example to the negatively charged teichoic acids (Antikainen, Kuparinen et al. 2007b, Weidenmaier, Peschel 2008, Kainulainen, Loimaranta et al. 2012).

The release of protein containing membrane vesicles (MVs) from the bacterial surface is another way for the bacterium to secrete proteins that do not have a canonical secretion signal sequence (Bonnington, Kuehn 2014). The release of MVs from the cell surface is a conserved feature in bacteria and has been demonstrated both in vitro and in vivo. It has been known for decades that MVs are released from Gram-negative bacteria. Recently, evidence has also accumulated indicating that Gram-positive bacteria use this export mechanism as well (Lee, Choi et al. 2009, Gurung, Moon et al. 2011, Lee, Lee et al. 2013, Thay, Wai et al. 2013). Natural MV production was found in SA a few years ago (Lee, Choi et al. 2009). The SA MVs share many features with the extensively studied outer membrane vesicles (OMVs) of Gram-negative bacteria; they are similarly spherical, bilayered, and closed membranous structures, albeit with slightly smaller diameters ranging from 20 to 100 nm (Figure 2) (Lee, Choi et al. 2008, Lee, Choi et al. 2009, Gurung, Moon et al. 2011).

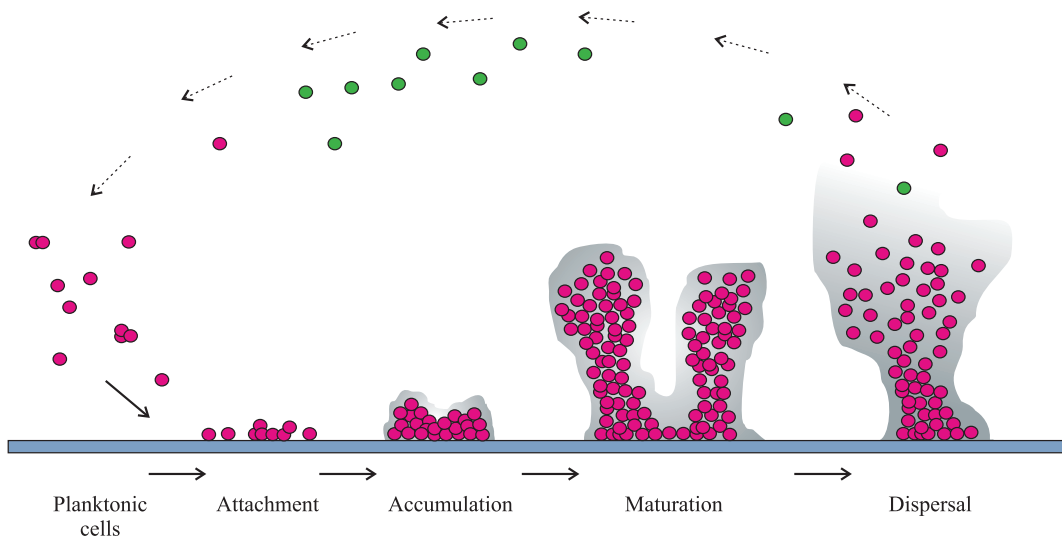
It has been proposed that the inclusion of proteins into MVs helps the bacteria to protect the proteins from extracellular proteolytic degradation, thus enhancing the long-distance protein delivery and ensuring an appropriate protein concentration at the target (Bonnington, Kuehn 2014). The numerous functions proposed for MV-based protein secretion include adherence, invasion, damage to host cells, immune response modulation, biofilm development, nutrient acquisition, interspecies cooperation, and protection against antibiotics and host defense mechanisms (Kulp, Kuehn 2010, Deatherage, Cookson 2012, Bonnington, Kuehn 2014). The MVs are known to deliver different toxins, enzymes and antigens, so they are commonly recognized as contributors of bacterial pathogenesis (Deatherage, Cookson 2012, Llobes, Bernadac et al. 2013).

### **1.2.3 Persistence through biofilm formation**

Since *SE* does not produce aggressive virulence determinants, the key factor in its pathogenicity in human infections is its ability to form biofilms. The biofilm is a structured, high-density and multilayered adherent growth mode of bacteria where the micro-organisms might be attached to a surface and to each other, and produce a protective extracellular polymer matrix - slime – consisting polymers such as polysaccharides, proteins and extracellular DNA. This growth mode enables quorum sensing cell-cell communication between the bacteria, protects the bacteria from being washed away and defends the bacterial community against environmental

stress, such as antibiotic drugs and the host immune defense system. This protection enables the biofilm-forming bacteria to survive and persist in the host both during normal colonization and in infectious states. (Costerton, Stewart et al. 1999, Mack, Davies et al. 2007, Otto 2008, Hoiby, Bjarnsholt et al. 2010)

The formation of biofilms proceeds through four stages: attachment, accumulation, maturation and dispersal (Figure 3) (Otto 2008, Fey, Olson 2010). The initial adherence to host matrix or biomaterial is nonspecific and hydrophobic in nature, but there are specific proteins called microbial surface components recognizing adhesive matrix molecules, MSCRAMMs, mediating this primary adhesion. These include autolysins AtlE and Aae, extracellular matrix binding proteins Embp and lipase GehD, that bind to host components such as fibronectin, fibrinogen, vitronectin and collagen. (Fey, Olson 2010, Otto 2008, Mack, Davies et al. 2007, Bowden, Chen et al. 2005) Furthermore, extracellular DNA (eDNA) released by SE has been implicated as a component contributing to the attachment phase by binding to appropriate surfaces such as polystyrene and glass (Qin, Ou et al. 2007).



**Figure 3.** Biofilm development. The main phases in biofilm development are attachment of the planktonic cells to the surface, bacterial accumulation, biofilm maturation, and dispersal. The green cells represent dispersed cells that have recently been described as unique intermediate phase in the bacterial life cycle (Chua, Liu et al. 2014). (Figure modified from: Otto, M. *Staphylococcus epidermidis*-the 'accidental' pathogen. *Nat Rev Microbiol.* 2009 Aug;7(8):555-67)

The next phase, accumulation, can be polysaccharide-, protein- and/or DNA-mediated. The polysaccharide intercellular adhesion (PIA)-mediated-biofilm formation is the most common way for SE to accumulate into biofilms, and this mechanism seems to be present in many disease-associated human strains (Fey, Olson 2010). PIA is encoded by the *ica* operon and is an extracellular polysaccharide mediating bacterial adherence and aggregation, contributing to virulence and defending the bacteria from the defenses mounted by the host. In addition to PIA, SE can also produce an exopolymer called poly- $\gamma$ -glutamic acid (PGA), which is an essential compound of the biofilm matrix and a key player in immune evasion in both commensal



and infectious lifestyles of *SE*. PGA protects *SE* against high salt concentrations and mediates resistance to antimicrobial peptides and phagocytosis, possibly interacting with PIA. (Mack, Fischer et al. 1996, Costerton, Stewart et al. 1999, Vuong, Voyich et al. 2004, Fey, Olson 2010) In the absence of the *ica* operon coding for PIA, PIA can be substituted with proteinaceous factors such as accumulation associated proteins Aap and biofilm-associated proteins bap/bhp in the biofilm formation, and in such situation, the biofilm is defined as being protein-mediated (Rohde, Burdelski et al. 2005, Tormo, Knecht et al. 2005). eDNA has also been proposed to function as a cell-to-cell connector in the biofilm accumulation phase of *SE* (Qin, Ou et al. 2007).

During the maturation phase, the bacteria shift their physiology towards anaerobic or microaerobic metabolism, and downregulate the synthesis of proteins, cell wall components and DNA. A mature biofilm contains bacteria that are in different metabolic states: some growing aerobically, some anaerobically, some are dormant and some are dead cells. (Fey, Olson 2010, Mack, Davies et al. 2007, Mah, O'Toole 2001) There are several proteins that are associated with biofilm maturation, such as accumulation-associated protein (Aap) and extracellular matrix binding protein (Embp). Nevertheless, the secreted surfactant peptides called phenol soluble modulins (PSMs) are the only biofilm maturation determinants for which there is *in vivo* evidence (Otto 2013b). In *SE*, PSMs and the Agr (accessory gene regulator) quorum-sensing system regulating the expression of PSMs have been identified as key players in the biofilm structuring, as well as in the final development stage i.e. biofilm detachment and dissemination (Wang, Khan et al. 2011, Periasamy, Joo et al. 2012, Otto 2013b). During the dispersal stage, individual bacterial cells can disengage from the biofilm and metastasize (Hoiby, Bjarnsholt et al. 2010, Otto 2013b). Recently, it has been demonstrated that in the bacterial life cycle, the dispersed cells represent unique intermediates between planktonic and biofilm lifestyles. In *Pseudomonas aeruginosa*, these dispersed cells have displayed unique phenotypes that differed from both planktonic and biofilm cells. For example, the dispersed cells were highly sensitive towards iron stress and were more virulent compared with the planktonic cells. (Chua, Liu et al. 2014)

The balance between the number of bacterial cells that are in the planktonic growth mode and those that are in the biofilm growth mode at a given time point is called phenotypic variation: a certain proportion of the population does not produce biofilm but stays planktonic. The rationale behind this is that the presence of diverse subpopulations increases the range of conditions in which the community as a whole can thrive. (Handke, Conlon et al. 2004, Fey, Olson 2010)

Even though the importance of biofilm formation in human nosocomial *SE* infections is currently accepted (Otto 2013b), there is no definite evidence that biofilm formation of CoNS has a role in the clinical outcome or severity of mastitis (Simojoki, Hyvönen et al. 2012, Tremblay, Lamarche et al. 2013). For example, in the study of Simojoki et al. (2012), where the biofilm-forming ability of almost 200 CoNS isolates from bovine mastitis was investigated, no association was detected between the ability to form biofilm / extracellular slime and the persistence of IMI or the severity of mastitis (Simojoki, Hyvönen et al. 2012). At the same time, slime production was found to be more common in persistent than spontaneously cured infections, and *SE* was producing slime more commonly than the other CoNS species studied. It has been hypothesized that even though biofilm formation does not contribute to the severity of IMI, it could promote the establishment and persistence of CoNS in the mammary gland, although the results for this proposal are indirect and speculative (Vanderhaeghen, Piepers et al. 2014). This hypothesis is in line with studies demonstrating that the biofilm-forming ability of *SA* promotes bacterial

colonization in the mammary gland and contributes to the higher bacterial resistance against treatment. These qualities in turn lead to persistent, albeit milder, infections than those caused by biofilm-negative strains. (Cucarella, Tormo et al. 2004, Clutterbuck, Woods et al. 2007)

#### **1.2.4 Resistance to antibiotics**

One of the problems in the treatment of *SE* infections is their resistance to many antibiotics. In particular, the resistance to methicillin is widespread in *SE* strains isolated from hospitals (methicillin-resistant *SE*, MRSE). It has been estimated that over 75% of *SEs* isolated from hospital environments around the world are resistant to methicillin, although there are extensive geographical variations in the frequency of resistant strains (Diekema, Pfaller et al. 2001, Stefani, Varaldo 2003). In addition to resistance against the widely used methicillin, there are strains that are resistant to several other groups of antimicrobial drugs, such as macrolides, aminoglycosides and tetracyclines. There are also strains that are multiresistant, and new mutations can lead to the birth of new resistances and combinations of multiresistance. (Carbon 2000, Rogers, Fey et al. 2009, Otto 2012) *SE* has also developed intermediate resistance to vancomycin, a phenomenon that is especially problematic because vancomycin is the most widely used antimicrobial agent to combat the severe infections caused by Gram-positive bacteria (Sanyal, Greenwood 1993, Jones 2006, Rogers, Fey et al. 2009). It has been hypothesized that *SE* can function as a reservoir and a source of antibiotic resistance genes that could be horizontally transferred to the more aggressive pathogen *SA*, converting it into antibiotic resistant strain such as MRSA (Otto 2013a). Although antibiotic resistance has spread widely among hospital-acquired *SE* isolates, it is considerably less frequent in community-acquired *SE* strains (Witte, Cuny et al. 2008, Rogers, Fey et al. 2009).

In addition, the formation of biofilm per se is an effective, non-specific mechanism of antibiotic resistance (Otto 2012). This is due to many attributes of the biofilm. For example, the biofilm matrix represents a physical and chemical barrier against penetrance for antibiotics, and it contains subpopulations of non-responding slow-growing and persister cells (Mah, O'Toole 2001, Stewart 2002). Thus, it must be considered that while the planktonic bacterial cells might be responsive to a certain treatment, its sessile, biofilm counterpart or the dispersed cells might not be treated by the same compound (Duguid, Evans et al. 1992, Van Mellaert, Shahrooei et al. 2012, Chua, Liu et al. 2014), which constitutes yet another obstacle in the fight against staphylococcal infections.

### **1.3 Genomic analyses of *SE***

The virulence factors of *SE* have been investigated by phenotypic and genotypic methods, but there are only a limited number of genomic studies focusing on *SE* strains of different environmental origins. Prior to this study, two complete genome sequences of *SE* had been reported, namely the genomes of the low-virulent ATCC12228 isolated from a healthy human host (Zhang, Ren et al. 2003) and the virulent RP62A isolated from a case of catheter-associated sepsis (Gill, Fouts et al. 2005).

The genome of ATCC12228 consists of one chromosome and six plasmids (Zhang, Ren et al. 2003). It has 2485 open reading frames (ORFs), and the chromosomal G+C ratio is 32.1%. The ATCC12228 genome was compared with the genome of *SA* strain N315, and this comparison revealed that ATCC12228 does not possess – apart from the  $\beta$ - and  $\delta$ -hemolysin genes - the exotoxin genes present in N315. Instead, it has genes for less invasive exoenzymes, fewer antibiotic resistance genes and it lacks the PIA-encoding *ica* operon. On the other hand,

ATCC12228 has a large repertoire of adhesin genes, which in the absence of the more aggressive pathogenicity determinants, can act as virulence factors in ATCC12228 when/if this strain is causing an opportunistic infection. The less invasive nature of ATCC12228, predicted from the genome data, has also been demonstrated in mouse and rat experimental infections. (Zhang, Ren et al. 2003)

The virulent strain RP62A has one chromosome and one plasmid, 2526 ORFs, and the chromosomal G+C ratio is 32.2% (Gill, Fouts et al. 2005). The genome of RP62A was compared with four *SA* strains (COL, Mu50, N315, MW2) and ATCC12228. This comparative analysis revealed that there are a core set of 1,681 ORFs common to all the studied strains. Variations between the pathogenic and resistance capabilities arise from specific genome islands, and in addition, the gene transfer between staphylococci and low-GC-content gram-positive bacteria can be a source of additional virulence factors. The study revealed that in *SE*, integrated plasmids contain e.g. genes that encode resistance to cadmium and species-specific surface proteins. PSMs and the *cap* operon encoding the polyglutamate capsule has also been postulated to be potential virulence factors of *SE*. A large part of the less invasive nature of *SE* as compared to that of *SA*, was explained by the lack of enterotoxin, exotoxin, leukocidin, and leukotoxin coding genes. The key factors in the stronger pathogenic capacity of RP62A as compared to the low-virulent ATCC12228 are considered to be the presence of the PIA-encoding *ica* operon and the adhesion- and biofilm-associated cell wall protein Bap/Bhp in RP62A, whereas these genetic elements are absent from the ATCC12228 genome. (Gill, Fouts et al. 2005)

In addition to the complete genomes of ATCC12228 and RP62A, there are several published *SE* draft genomes (Table 1). For example, the study of Conlan et al. (2012) revealed that the existence of the formate dehydrogenase gene (*fdh*) differentiates the commensal *SE* strains from the nosocomial strains. Therefore, *fdh* could be used as a marker gene for commensalism. (Conlan, Mijares et al. 2012) In the study of Madhusoodan et al. (2011), an *S. epidermidis* pathogenicity island (SePI) was found for the first time from a clinical *SE* isolate strain FRI909. This SePI encodes staphylococcal enterotoxin C3 and staphylococcal enterotoxin-like toxin L, which is in line with the fact that the majority of virulence factors, superantigens and toxins of *SA* are encoded by *S. aureus* pathogenicity islands, SaPIs (Novick, Subedi 2007). Al-Mahrous et al. (2011), in turn, examined the *SE* strain A487 and identified a hemolysin-like peptide that exerts antibacterial activity against other *Staphylococcus* strains, including several MRSA strains.

**Table 1.** *The published SE draft genomes, strains and characteristics.*

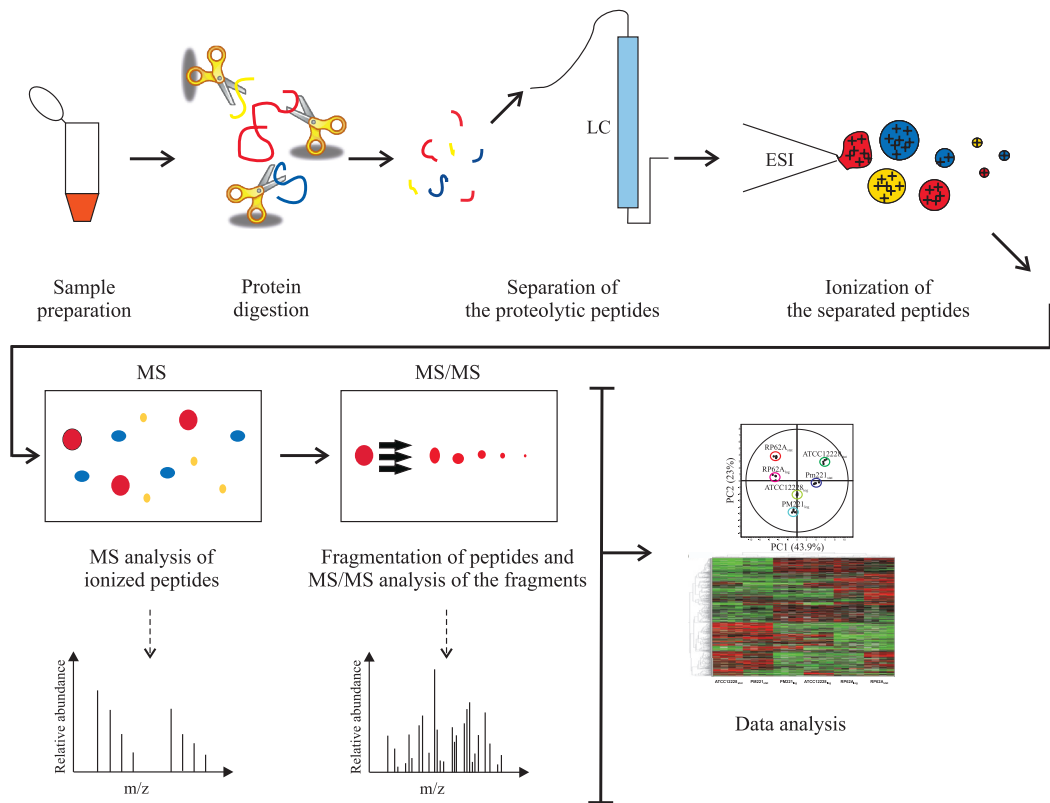
Name of the strain	Isolated from	Characteristics	Reference
ET-024	biofilm on an endotracheal tube of a mechanically ventilated patient	resistant to methicillin	(Vandecandelaere, Van Nieuwerburgh et al. 2014)
UC7032	cured meat	heteroresistant to glycopeptide antibiotics	(Gazzola, Pietta et al. 2013)
AU12-03	intravascular catheter	may form biofilms, resistance to fluoroquinolone and beta-lactam antibiotics	(Zhang, Morrison et al. 2012)
FRI909	human source	SePI in genome, produces enterotoxin C	(Madhusoodanan, Seo et al. 2011)
A487	human source	Inhibitory activity against MRSA	(Al-Mahrous, Jack et al. 2011)
CSUR P278	native-aortic-valve endocarditis		(Fournier, Gouriet et al. 2013)
NIH04003, NIH04008, NIH05001, NIH05003, NIH05005, NIH051475, NIH051668, NIH06004, NIH08001, NIHLM001, NIHLM003, NIHLM008, NIHLM015, NIHLM018, NIHLM020, NIHLM021, NIHLM023, NIHLM031, NIHLM037, NIHLM039, NIHLM040, NIHLM049, NIHLM053, NIHLM057, NIHLM061, NIHLM067, NIHLM070, NIHLM087, NIHLM088, NIHLM095	21 commensal strains from healthy humans and 9 nosocomial strains from human infections	considerable diversity between commensal isolates; nosocomial isolates exhibited large-scale rearrangements and single-nucleotide variation	(Conlan, Mijares et al. 2012)
APO27, APO35, CIM40, MC16, MC19, Scl19, Scl25, CIM37, CIM28, WI05, WI09	11 strains from wild mice	83.6% to 92.2% of the proteins have homologs in ATCC12228 and/or RP62A genomes	(Wang, Kuenzel et al. 2014)

These genomic studies provide some hypotheses about the mechanisms by which *SE* can be transformed from a commensal inhabitant to an invasive pathogen, but nonetheless proteome-level information is required in order to confirm and complement these hypotheses.

## **1.4 Proteomic analyses of *SE***

The proteome is the whole set of proteins that are synthesized by a given cell under certain conditions at a particular time point. The term proteomics refers to a set of technologies that are used for the analysis of proteomes, including protein identifications, modifications, localizations, dynamics and abundances. It is a group of high-throughput methods that are largely based on the use of mass spectrometry (MS) for the identification and quantitation of proteins and modifications. Previously, 2-DE (two-dimensional gel electrophoresis) was most often considered to be the method of choice in proteomics. In 2-DE, the first dimension of protein separation is isoelectric focusing (IEF) where the proteins are separated based on their isoelectric points (pIs), and the second dimension is SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) which additionally separates the proteins according to their molecular weights (MWs). The resulting two-dimensional gel is visualized and the protein spots of interest can be cut out and identified by MS. Recently, proteomics has moved away from the use of 2-DE towards the application of 1-DE (SDS-PAGE) separation methods and in-liquid-digestion prior to LC-MS/MS (liquid chromatography combined with tandem mass spectrometry) analysis, and this combination is now the most popular method in proteomics (Tyers, Mann 2003, Walther, Mann 2010, Lamond, Uhlen et al. 2012).

The general work-flow of LC-MS/MS-based proteomics consists of i) sample preparation (e.g. organelle fractionation, labelling, gel-based protein separation etc.), ii) proteolytic protein digestion, iii) separation and ionization of the proteolytic peptides by LC and electrospray ionization (ESI), respectively, iv) analysis of the ionized peptides with MS, v) fragmentation of the selected peptides and the analysis of the fragments with MS/MS, and vi) data-analysis, including identification and possible quantification as well as bioinformatic analysis (Figure 4) (Walther, Mann 2010).



**Figure 4.** The general work-flow of LC-MS/MS-based proteomics. The main stages are sample preparation, proteolytic protein digestion, separation and ionization of the proteolytic peptides, MS- and MS/MS-analysis, and data-analysis.

Although *SE* is a significant nosocomial micro-organism as well as a bovine pathogen, there are only few proteomic studies of *SE*, and most of these studies have been 2-DE -based applications (Table 2). Even though 2-DE is useful for visualizing metabolic pathways and responses to different stimuli, it does have its limitations for example in detecting the hydrophobic IMPs and low abundant proteins (Hecker, Antelmann et al. 2008). These proteomic studies have mostly targeted specific phenomena or specific proteome fractions instead of providing a comprehensive proteomic overview or profiling. They have tended to focus on the effects of growth phase and quorum-sensing system mutations on the proteome (Batzilla, Rachid et al. 2006), on the effect of different concentrations of an SPase inhibitor on the stationary-phase secretome (Powers, Smith et al. 2011), on the immunoreactive or serum binding cell wall-associated proteins (Sellman, Howell et al. 2005), and on the differences between an invasive (RP62A) and a commensal (ATCC12228) *SE* strain (Yang, Li et al. 2006). The last-mentioned study resulted in the generation of 2D-gel reference maps of these two strains, but since the study focused on the differentially expressed proteins, only those proteins were identified (a total of 155 identifications).

**Table 2.** *The proteomic fractions studied, the proteomic methods used and the central findings of previous proteomic studies of SE.*

Study	Proteins studied	Protein separation	Dye/label/probe	MS analysis	Central findings
Batzilla et al. (2006)	extracellular	2-DE	DIGE	MALDI-TOF MS	<i>agr</i> mutant expressed significantly lowered amounts of several extracellular proteins
	cytoplasmic	2-DE	CBB G-250	MALDI-TOF MS	<i>agr</i> mutant altered the regulation of virulence and amino acid / carbohydrate metabolism
Powers et al. (2011)	secreted	2-DE	SYPRO-Ruby	LC-MS/MS (LTQ linear ion trap)	SPase inactivation decreased the secretion of several proteins
Sellman et al. (2005)	cell wall -associated	2-DE + blot	sera and serum proteins	MALDI-TOF MS and LC-MS/MS (ESI-QUAD-ion trap)	the discovery of five candidate vaccine candidates
Yang et al. (2006)	extracellular	2-DE	Silver staining	MALDI-TOF MS	proteome maps of RP62A and ATCC12228 and identification of 155 differentially expressed proteins
	membrane	2-DE	Silver staining	MALDI-TOF MS	



Batzilla et al. (Batzilla, Rachid et al. 2006) investigated how a mutation in the quorum-sensing accessory gene regulatory system (Agr) could affect protein synthesis. This group used 2-DE with Coomassie staining (extracellular proteins) and DIGE labelling (cytoplasmic proteins) combined with MALDI-TOF/MS (matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry) to compare the extracellular and cytoplasmic proteins of *SE* strains 567 (wild type) and 567-1 (*agr* mutant). Their results revealed that both the Agr system and the growth phase could affect the expression of surface proteins, with 14 of these differentially expressed proteins being identified. The *agr* mutant expressed significantly reduced amounts of several extracellular proteins, such as serine proteases SspA, autolysin AtlE, lipase GehD and phenol soluble modulins. They identified 51 proteins from the cytoplasmic fraction, and observed that the inactivated *agr* led to a number of alterations in the regulation of virulence as well as in the metabolism of amino acids and carbohydrates. (Batzilla, Rachid et al. 2006)

In the study of Powers et al. (Powers, Smith et al. 2011), the focus was on the type I signal peptidase and protein secretion in *SE*. The Sec-based protein export system was blocked with different concentrations of arylomycin C<sub>16</sub>, an SPase inhibitor, in strain RP62A during its stationary growth phase. They utilized a combination of 2DE and LC-MS/MS, and identified 11 proteins with decreased secretion i.e. that were dependent on SPase activity. Nine of these proteins had a canonical N-terminal SPase recognition site but the remaining two did not possess this sequence. The nine proteins with the canonical recognition site included autolysin AtlE, immunodominant staphylococcal antigen IsaA, serine protease SspA, cysteine protease SspB, and secreted lipases GehC and GehD. The two proteins with a non-canonical recognition site were lipoteichoic acid synthase LtaS and a transmembrane protein BlaR1, a component of a two-component response regulator involved in the sensing of  $\beta$ -lactam antibiotics (Kobayashi, Zhu et al. 1987). (Powers, Smith et al. 2011)

Sellman et al. (Sellman, Howell et al. 2005) focused on the bacterial proteins that are expressed upon transition into the bloodstream as well as on the proteins that interact with the host. The bacteria were grown in serum to mimic the environment of the bloodstream. The cell wall-associated proteins were isolated, separated with 2-DE, and transferred to nitrocellulose membranes. The proteins were then analyzed for reactivity with sera derived from rabbits that had been immunized with live *SE* and with biotin-labelled serum proteins eluted from the surface of bacteria grown in rabbit serum. The group identified 29 immunoreactive or serum binding proteins. Twenty-seven of these proteins were expressed in *E. coli*, and these recombinant proteins were then used to immunize mice by vaccination. Five of the recombinant proteins induced a statistically significant reduction in the numbers of bacteria recovered from the spleen or bloodstream of the infected mice. These were Na<sup>+</sup>/H<sup>+</sup> antiporter, alanine dehydrogenase, acetyl-CoA acetyltransferase, lipotease ligase and cysteine synthase, and they were considered as candidate vaccine antigens. (Sellman, Howell et al. 2005)

Yang et al. (Yang, Li et al. 2006) conducted a comparative proteomic analysis of two *SE* strains, the invasive strain RP62A and the commensal strain ATCC12228 using 2DE and MS in an attempt to unravel the key proteins in pathogenicity. The overall proteome maps were similar in both strains, but there were 168 proteins spots exhibiting differential expressions and almost all (155) of these could be identified. The differentially expressed proteins were involved in carbohydrate metabolism, sugar binding, lipid degradation and amino acid binding. Furthermore, the levels of accumulation-associated protein (Aap), an important protein in biofilm formation, were lower in the non-biofilm forming ATCC12228 in comparison to the concentrations in the biofilm forming RP62A. (Yang, Li et al. 2006)



## 2 AIMS OF THE STUDY

*Staphylococcus epidermidis* is a major nosocomial and mastitis-causing bacterium with significant medical and veterinary relevance. The aim of this study was to create proteomic profiles of three strains of *SE*, one representing a low-virulent, one a virulent and the third being an animal strain (ATCC12228, RP62A and PM221, respectively), to identify the factors that differentiate the commensal lifestyle from its pathogenic counterpart, and to reveal the mechanisms behind *SE*'s virulence, survival and adaptation.

The more detailed aims were:

- To sequence the entire genome of the bovine mastitis associated strain (PM221) and to compare its genome sequence with those of the published *SE* genomes (I)
- To study the protein expression dynamics in the logarithmic and stationary growth phases of PM221, ATCC12228 and RP62A (I)
- To characterize the expressed total proteomes (II) and surfacomes (I) of PM221 and ATCC12228, and the exoproteomes (III) of PM221, ATCC12228 and RP62A
- To complement the genome and proteome findings by using an *in vivo* infection model (I)

## 3 MATERIALS AND METHODS

### 3.1 *SE* strains (I, II, III)

The *SE* strains used in the genome- and proteome-level analysis were PM221, a strain causing bovine mastitis isolated from a persistent IMI (Taponen, Koort et al. 2007), and two human *SE* strains, non-biofilm-forming ATCC12228 (Zhang, Ren et al. 2003) with low infectious potential isolated from a healthy human host, and a biofilm-positive, multi-resistant RP62A (Gill, Fouts et al. 2005) that is able to cause chronic human infections and which had been isolated from catheter-associated sepsis.

### 3.2 Genome sequencing, assembly and annotation (I)

Genomic and plasmid DNAs of PM221 were extracted from planktonic cultures. The genome of PM221 was sequenced, and the reads were assembled into contigs, most of them mapped to the genome of ATCC12228, and the gaps were closed. Protein coding sequences were predicted with Glimmer and RAST (Rapid Annotation using Subsystem Technology), and the automated annotations obtained by RAST were manually adjusted based on information from different databases (e.g. KEGG, TIGR, Prosite and InterPro and COG) Prophages, whole-genome nucleotide alignments, IS elements and RNA genes were predicted using several tools (Prophinder, BLAST, RAST).

### 3.3 Genome and gene comparisons (I)

Orthologs and in-paralogs among the genes of PM221, ATCC12228 and RP62A were identified using two complementary tools (BLASTP, OrthoMCL) and on the basis of protein sequences from 36 genomes of interest. Nine different species were used in the genome comparison: *SE*, *SA*, *Staphylococcus capitis*, *Staphylococcus carnosus*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus saprophyticus*, and *Staphylococcus warneri* (Table 3). The phylogenetic tree was constructed using OrthoMCL, Muscle, GBLOCKS and PhyML. The whole genome and plasmid sequences of PM221 were compared with the published genome sequences of ATCC12228 and RP62A.

**Table 3.** The *Staphylococcus* strains used in the pan-genome analysis. Protein sequences were downloaded from <ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/>

Strains	
<i>Staphylococcus epidermidis</i> ATCC12228	<i>Staphylococcus aureus</i> Mu3
<i>Staphylococcus epidermidis</i> BCM-HMP0060	<i>Staphylococcus aureus</i> Mu50
<i>Staphylococcus epidermidis</i> M23864:W1	<i>Staphylococcus aureus</i> MW2
<i>Staphylococcus epidermidis</i> M23864:W2	<i>Staphylococcus aureus</i> N315
<i>Staphylococcus epidermidis</i> PM221	<i>Staphylococcus aureus</i> NCTC 8325
<i>Staphylococcus epidermidis</i> RP62A	<i>Staphylococcus aureus</i> Newman
<i>Staphylococcus epidermidis</i> SK135	<i>Staphylococcus aureus</i> RF122
<i>Staphylococcus epidermidis</i> W23144	<i>Staphylococcus aureus</i> ST398
<i>Staphylococcus aureus</i> 04-02981	<i>Staphylococcus aureus</i> TW20
<i>Staphylococcus aureus aureus</i> MRSA252	<i>Staphylococcus aureus</i> USA300 FPR3757
<i>Staphylococcus aureus aureus</i> MSSA476	<i>Staphylococcus aureus</i> USA300 TCH1516
<i>Staphylococcus aureus</i> COL	<i>Staphylococcus capitis</i> SK14
<i>Staphylococcus aureus</i> ED133	<i>Staphylococcus carnosus</i> TM300
<i>Staphylococcus aureus</i> ED98	<i>Staphylococcus haemolyticus</i> JCSC1435
<i>Staphylococcus aureus</i> JH1	<i>Staphylococcus hominis</i> SK119
<i>Staphylococcus aureus</i> JH9	<i>Staphylococcus lugdunensis</i> HKU09-01
<i>Staphylococcus aureus</i> JKD6008	<i>Staphylococcus saprophyticus</i> ATCC 15305
<i>Staphylococcus aureus</i> JKD6159	<i>Staphylococcus warneri</i> L37603

### 3.4 Experimental infection (I)

One udder quarter of two dairy cows was infected with ATCC12228, with another udder quarter being used as a control. Milk samples and clinical data were collected for two weeks, and bacterial counts, somatic cell counts (SCC), and milk N-acetyl- $\beta$ -D-glucosaminidase (NAGase) activity in the milk were determined. Also the local and systemic clinical signs were also recorded.

### 3.5 Growth phases and proteome subfractions (I, II, III)

The bacterial cells were withdrawn from growth phases ranging from the mid-exponential to post-exponential stages (Table 4) since it is known that the production of virulence factors is maximal and the cell lysis minimal during this period of bacterial growth (Fischetti, Novick et al. 2000).

**Table 4.** Bacterial growth stages from which the proteins samples were derived and the proteomic sub fractions collected and analyzed.

	I			II	III
	2D DIGE	Surfacome shaving		Total proteome	Exoproteome
The phase during which the cells were collected	mid-exponential and onset of stationary	mid-exponential		post-exponential	late-exponential
OD600	~1.0±0.1 and ~4.0±0.1	~0.9-1.0 (aerobic)	~0.7-0.8 (microaerophilic)	~4.0 ± 0.1	~1.9 ±0.2
Fraction collected for proteomic analysis	whole cells	trypsin-digested surfacome proteins		whole cells	supernatants

### 3.6 Proteomic work-flow – protein and peptide separation, analysis and identification (I, II, III)

In the 2D DIGE analysis of the total proteomes of PM221, ATCC12228 and RP62A (I), the extracted and purified proteins were labelled with Cy2, Cy3, or Cy5 dyes. The labeled proteins were first separated using IEF, and then with SDS-PAGE. The gel images were analyzed with the DeCyder 2D 7.0 software, and the statistical analyses showed strain and growth-stage specific changes in protein abundances. The selected spots fulfilling the chosen criteria for different abundances were cut out for in-gel digestion with trypsin followed by LC-MS/MS identification using Ultimate 3000 nano-LC and QSTAR Elite hybrid quadrupole TOF mass spectrometer with nano-ESI ionization.

In the analysis of the surfacome proteins of PM221 and ATCC12228 (I), the surface-attached proteins were released by trypsin-shaving from intact living cells. The trypsin-digested peptides were purified with ZipTips ( $\mu$ C18), and the tryptic peptides were identified using LC-MS/MS.

The extracted and purified protein samples from the whole cell lysate and supernatant in the total proteome cataloging (II) and exoproteome cataloging (III), respectively, were analyzed with GeLC-MS/MS (protein separation with one-dimensional gel electrophoresis coupled with protein identification with liquid chromatography – tandem mass spectrometry). First, the proteins were separated with SDS-PAGE, the gel lanes were sliced into fractions, and the proteins were reduced, alkylated and in-gel digested with trypsin. The resulting tryptic peptides were extracted from the gel, and analyzed with LC-MS/MS.

The acquired MS/MS data were search against in-house databases with two search engines – Mascot and Paragon – except for the experiment with 2D DIGE analysis (I), where only Mascot was used. The in-house databases were composed of the open reading frame sets (ORFs) of PM221 (2530 entries), ATCC12228 (2485 entries) and RP62A (2526 entries). The Compid tool (Lietzen, Natri et al. 2010) was used to parse significant hits from Mascot and Paragon searches.

### 3.7 Computational proteome analyses (I, III)

Several prediction algorithms were used to characterize the proteins of the three studied *SE* strains. These algorithms were used to acquire several parameters e.g. theoretical molecular weights (MW), isoelectric points (pI), GRAVY values (grand average hydropathy), cellular locations, GO (gene ontology) annotations, and COG (clusters of orthologous groups) categories of proteins, as well as the presence of potential signal sequences, transmembrane domains, lipoboxes, and cell wall anchor motifs.

### 3.8 Phenotypic analyses (I, II, III)

The catalase activity was measured by mixing cell samples with Triton X-100 and hydrogen peroxide. After mixing and incubation, the height of the O<sub>2</sub>-forming foam, reflecting the catalase activity in the test tube, was visually compared. A detailed description of the method is presented in study I.

The tendency to form small colony variants (SCVs) was tested in a plating assay. The three *SE* strains were cultured for 24 h, 36 h and 48 h in tryptic soy broth (TSB). The cell samples withdrawn at the three time points and then serially diluted and plated. The plates were incubated at 37°C under aerobic conditions to obtain colonies, and the sizes and morphologies of these colonies were visually assessed (I).

The ability of PM221 to grow as biofilm was tested by evaluating protein- and DNA-mediated adherent growth. ATCC12228 was used as non-biofilm- and RP62A as biofilm-forming controls. The strains were grown on a polystyrene support, and the protein-mediated adherent growth was tested using Proteinase K, whereas the DNA-mediated adherent growth was assessed using DNaseI. The absorbance of the crystal violet stained biofilms were measured at 590 nm with a PerkinElmer Victor3 multilabel microtiter plate reader (II).

The lipolytic activity was estimated with the tributyrin plate assay, where the tributyrin agar plate shows a zone of triglyceride hydrolysis due to esterase/lipase activity of the bacteria (II).

The  $\beta$ -lactamase activity of culture supernatants was tested by using  $\beta$ -lactamase susceptible nitrocefin as the substrate, and the urease activity was evaluated by using urea as the substrate. The hydrolysis of both substrates was monitored after 20 h of incubation at 37 °C at 560 nm (Ure) and 490 nm (Bla) (III).

### 3.9 Enrichment of membrane fraction

The membrane vesicle fraction was enriched from the culture media by ultrafiltration with 100 kDa cut-off membrane units. The protein composition of culture media, flow-throughs and concentrated retentates were assessed by SDS-PAGE and SYPRO-Orange staining (III).

## 4 RESULTS

This study was designed to answer the need for comprehensive genome- and proteome-level data from *SE* in order to identify the factors that would differentiate a commensal lifestyle from its pathogenic counterpart, as well as the mechanisms behind *SE*'s virulence, survival and adaptation. Three *SE* strains were studied: the recently isolated IMI causing bovine strain PM221 (Taponen, Koort et al. 2007), the human strain ATCC12228 with a low infection potential (Zhang, Ren et al. 2003), and the sepsis-associated virulent human strain RP62A (Gill, Fouts et al. 2005).

### 4.1 Genome-level and total proteome analyses revealed the similarity between PM221 and ATCC12228 (I, II)

The PM221 genome comprises a single 2490012 bp long circular chromosome (GC content = 32 %), that encodes for 2393 proteins. It has additionally four plasmids that encode 116 proteins. The genome-level profiling and comparison between PM221, ATCC12228 and RP62A (I) revealed that PM221 and ATCC12228 are very similar (I/ Fig. 1); the overall gene organization of PM221 resembles more ATCC12228 than that of RP62A, and the phylogenetic analysis showed that the bovine strain is also evolutionary closer to ATCC12228 than to RP62A. One distinct difference noted between PM221 and the human strains was the high number (almost 600) of genes with paralogues as compared to the two human strains (286 and 252 genes with paralogues in ATCC12228 and RP62A, respectively).

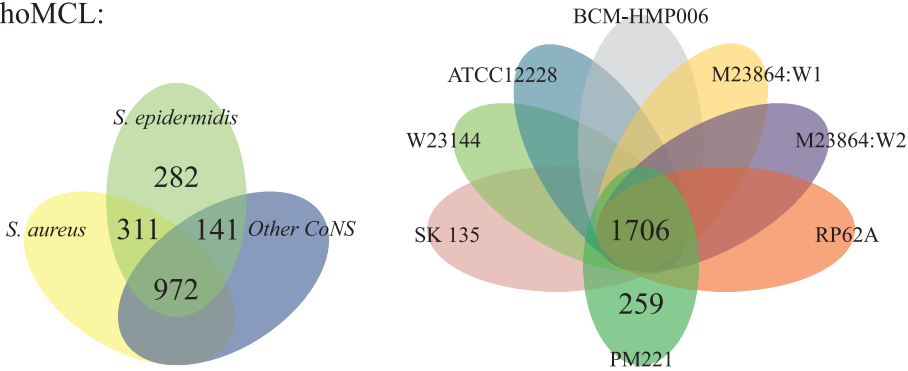
The PM221 genome has characteristics associated with a commensal lifestyle, for example it possesses the formate dehydrogenase, *fdh*, a marker gene for commensalism (Conlan, Mijares et al. 2012), and it lacks the clustered regularly interspaced short palindromic repeat (CRISPR) element that is thought to confer resistance against phages (Barrangou, Fremaux et al. 2007). The lack of the CRISPR element has presumably promoted the acquisition of prophage-like elements and specific genes coding for phage proteins, and one of the prophage elements was integrated into a genomic island encoding two superantigens. Arginine catabolic mobile element (ACME) that is recognized as a putative pathogenicity island and an important virulence factor in some non-biofilm-producing *SE* strains (Diep, Stone et al. 2008) was identified only in the PM221 and ATCC12228 genomes. The genome of PM221 harbors also other pathogenic attributes such as proteases, lipases and hemolysins. One of the proteins associated with biofilm formation, the accumulation associated protein (Aap), was located in a plasmid in PM221 whereas it was chromosomally located in the human strains.

The genetic resemblance of PM221 and ATCC12228 was further confirmed in a whole-genome comparison (unpublished data) conducted with 36 *Staphylococcus* genomes using two orthologue prediction tools, OrthoMCL and RBBH. This comparison revealed that of all the strains studied (8 strains of *SE*, 21 strains of *SA*, and *S. capitis*, *S. carnosus*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. saprophyticus*, and *S. warneri*, one strain of each) the genome of PM221 most resembled the genome of ATCC12228 (Figure 5), and the genomes of these two strains shared 2233 genes with each other. According to the RBBH analysis that utilizes more stringent criteria for unique genes than OrthoMCL, there were 1274 conserved genes that were found in each of the 36 studied genomes. There were 172 genes that were common to all eight *SE* strains, but not found in the shared genomes of the *SA* strains or the shared genomes of other CoNS strains. Additionally, *SE* shares more genes with *SA* (239) than with other CoNSs strains studied (180). The preliminary analysis suggested that genes shared by all *SE* and *SA* strains,

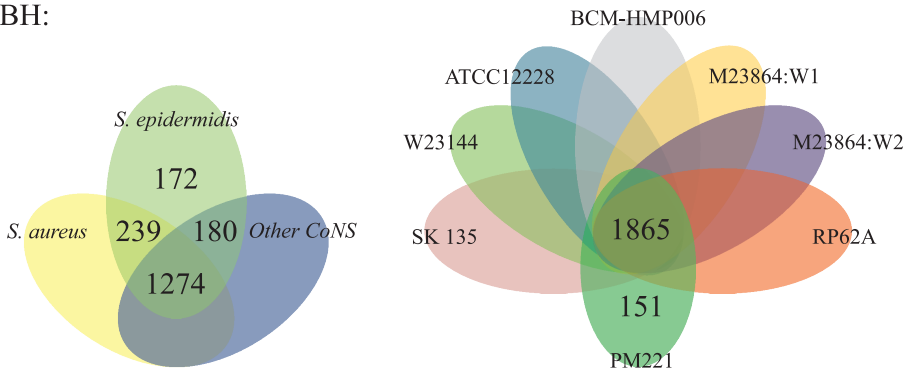
but not all CoNS strains, include a high number of genes for transporter proteins, whereas *SE* strains and CoNS strains tend to share genes for DNA- and ribosome-associated proteins. The *SE*-specific genes common to all the studied *SE* strains include genes coding for capsule biosynthesis-associated proteins, resistance-related proteins and transcriptional proteins.

When the *SE* genomes were compared with each other, there was a total of 1865 conserved genes in all eight *SE* strains whereas the number of genes found only in PM221 was 151. The genes that were found only from the genome of PM221 included several phage-related genes, truncated transposases and integrases.

#### OrthoMCL:



#### RBBH:



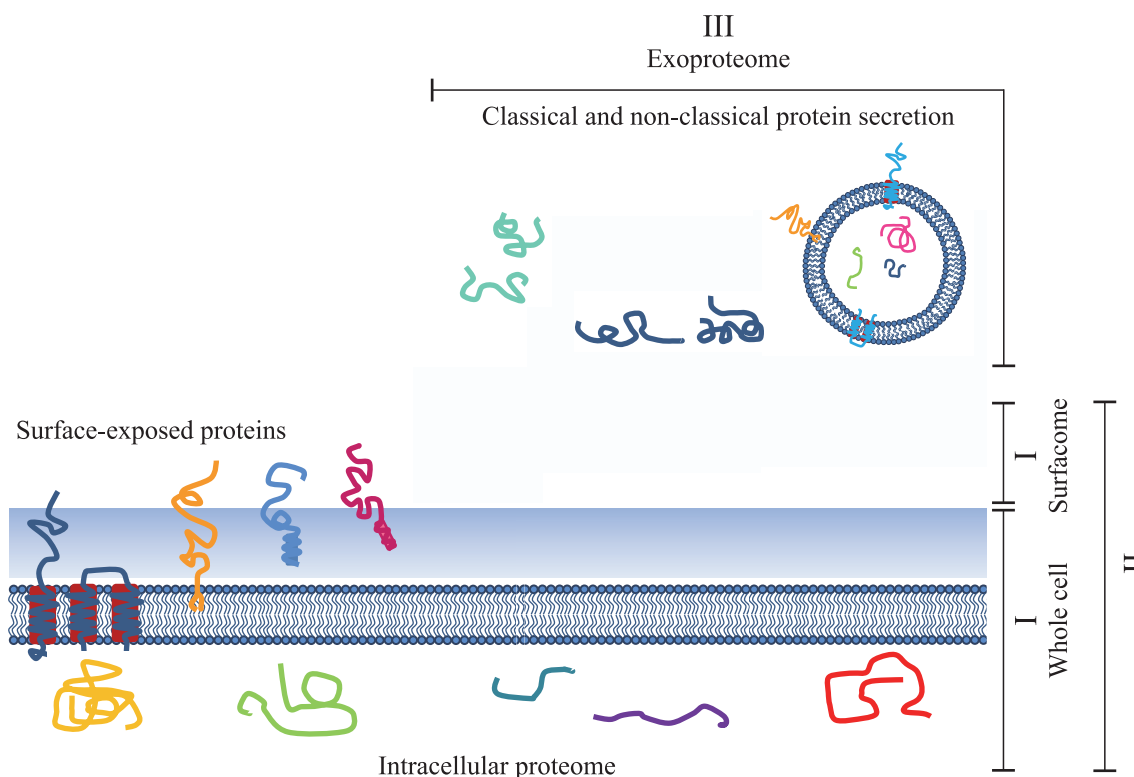
**Figure 5.** On the left: Pan-genome comparison of 36 *Staphylococcus* genomes. On the right: Comparison of the genomes of eight *SE* strains (PM221, SK 135, W23144, ATCC12228, BCM-HMP006, M23864:W1, M23864:W2, RP62A). The numbers of conserved and unique genes are indicated. The orthologue prediction tools used were OrthoMCL (the two upper Venn diagrams) and RBBH (the two lower Venn diagrams).

## 4.2 Over 50% of the predicted proteome could be identified (I, II, III)

Despite of the genetic similarity between PM221 and ATCC12228, these two strains exhibit different phenotypes (e.g. biofilm-formation, different infection potentials, and adaptation to different environmental niches). Proteomic approaches were used to unravel these differences

and to identify the factors that would differentiate a mildly infectious lifestyle (PM221) from the non-infectious lifestyle (ATCC12228) as well as distinguishing from the infectious lifestyle (RP62A).

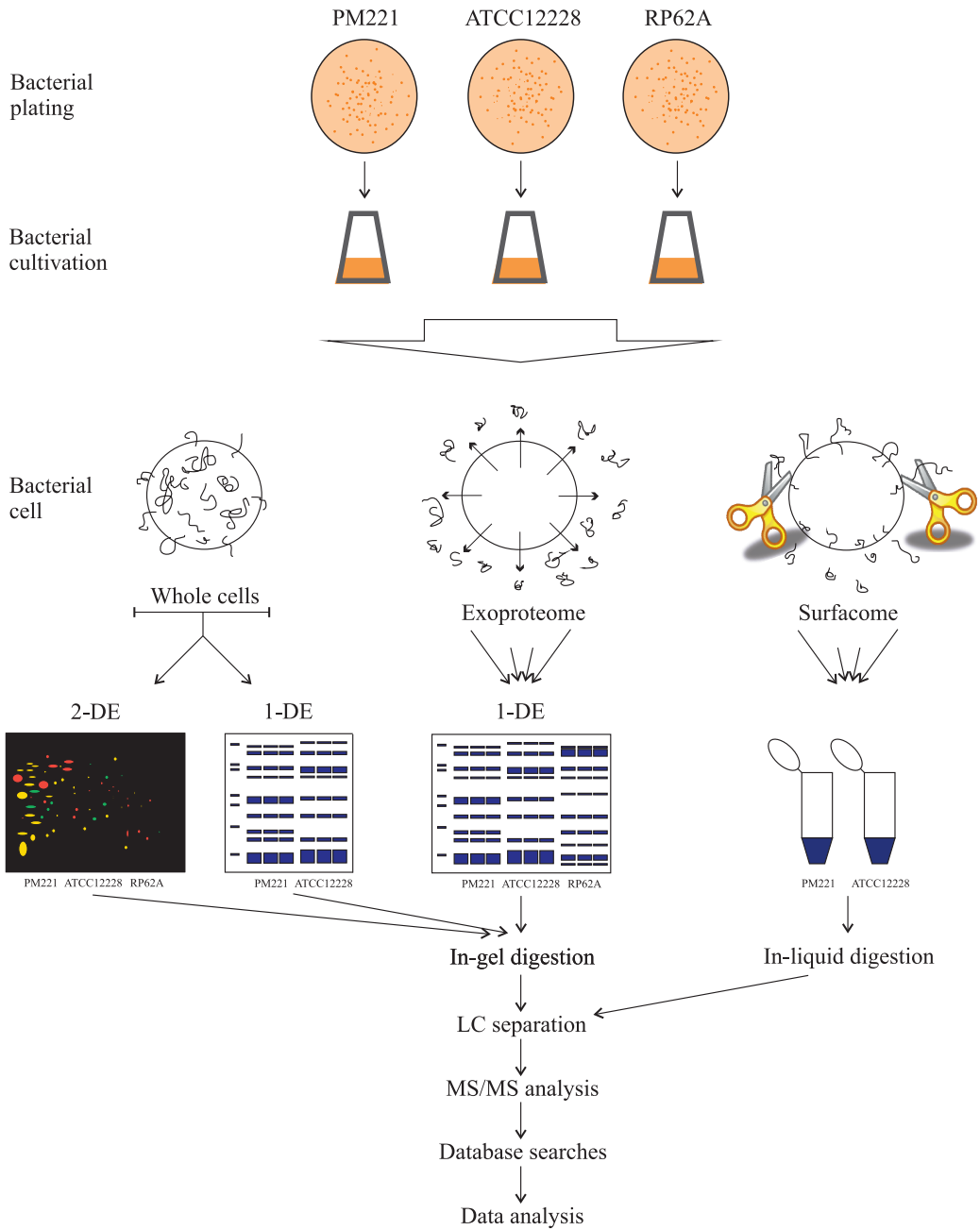
In the proteomic analyses, all proteomic subfractions that can be isolated from Gram-positive bacteria were covered; cytosolic proteome (I and II), membrane- and cell wall-associated proteins (I and II), and exoproteome i.e. secreted proteins (III) (Figure 6).



**Figure 6.** Sub-proteomic fractions that were covered in the three proteomic studies (I, II, III).

The proteomic analyses were i) the comparative analysis of the three SE strains (PM221, ATCC12228 and RP62A) using 2D DIGE (I), ii) the cell-surface shaving proteomics of PM221 and ATCC12228 (I), iii) the GeLC-MS/MS analysis for a global proteome profiling and comparative proteome analysis of PM221 and ATCC12228 (II), and iv) GeLC-MS/MS analysis of the exoproteomes of PM221, ATCC12228 and RP62A (III) (Figure 7).



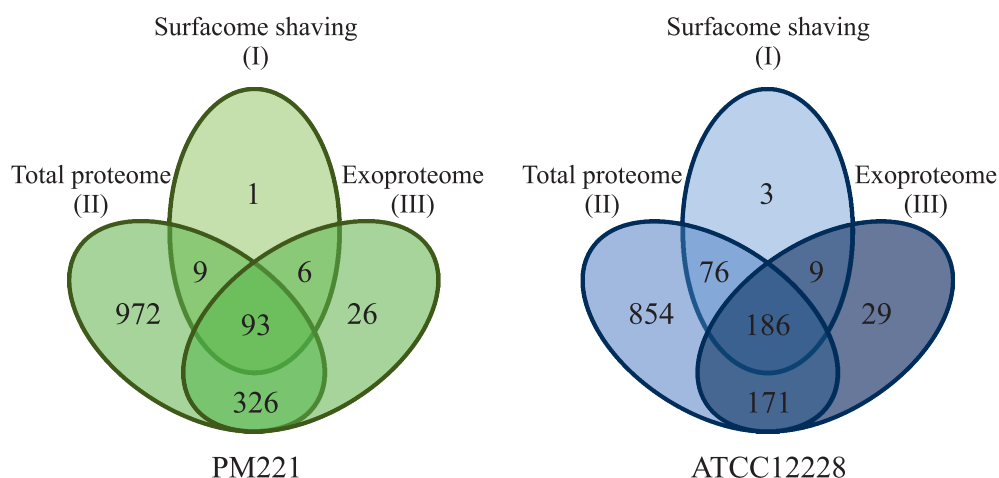


**Figure 7.** The work-flows of the three proteomic studies (I, II, III).

In the total proteome cataloging (II) done by using GeLC-MS/MS, it was possible to identify 1400 and 1287 proteins from PM221 and ATCC12228, respectively, accounting for over 50% of the predicted proteomes. Since only around 50-80% of the bacterial genome is expressed under specific condition and growth phase (Hecker, Antelmann et al. 2008), the coverage of the expressed proteome exceeds well over 50%, representing the first extensive proteome survey of *SE*. This large amount of identifications also strengthens the genomic annotations of these strains.

Almost 1200 of the identified proteins were identified from both strains, e.g. they were strain-shared identifications. In addition to these proteins, there were 200 (PM221) and 74 (ATCC12228) identified proteins that had a genetic equivalent in both strains but were identified only from one strain, e.g. they were strain-specific identifications. Although these two strains are genetically very similar, there were 289 (PM221) and 260 (ATCC12228) unique genes that were not found in the other strains genome, and it was possible to identify 39 (PM221) and 42 (ATCC12228) of these unique proteins. More surface-associated proteins could be identified from PM221 than from ATCC12228, accounting for over 30% of the predicted surfacome. In contrast, in the trypsin shaving experiment more surfacome proteins were identified from ATCC12228 than from PM221. In the exoproteome analysis, over 330 of the identified exoproteins were identified from all three strains, whereas the numbers of unique and strain-specific identifications were 48, 22, and 98 in PM221, ATCC12228 and RP62A, respectively.

Taken together (I, II, III), by using different proteomic approaches, it was possible to identify a total of 1433 proteins from PM221 and 1328 proteins from ATCC12228 (Figure 8). The 33 proteins identified from PM221 in surfacome shaving (I) and exoproteome characterization (III) included virulence-associated proteins such as Geh2-lipase, a metalloproteinase precursor SepA, and cell-wall anchored protein SesC that were not identified in the total proteome study. The total of 41 proteins identified from ATCC12228 in surfacome shaving and exoproteome characterization included adhesion- and resistance-promoting proteins such as penicillin binding protein 4, fibrinogen-binding protein SdrG, surface protein SesH, TcaA protein which is involved in glycopeptide resistance, elastase binding SepA, SesC, and fibrinogen-binding SdrF.



**Figure 8.** The number of proteins identified from PM221 and ATCC12228 in total. The Venn diagrams display the number of unique and shared identifications from the surfacome shaving experiment (I), total proteome cataloging (II) and exoproteome characterization (III).

### **4.3 The protein expression profiles of PM221 and ATCC12228 are more alike while RP62A displays more virulence-related attributes (I)**

The total proteome dynamics of the three strains were studied with 2D DIGE based proteomics (I). In this experiment, protein samples from logarithmic and stationary growth phase cell lysates were purified, labelled with fluorescent dyes, separated with 2DE and visualized. The data generated by DeCyder analysis was further investigated with statistical techniques (Principal Component Analysis, PCA and Hierarchical Clustering Analysis, HCA) which revealed that the protein expression patterns of PM221 and ATCC12228 in both growth phases clustered close to each other, whereas RP62A formed a separate cluster. This indicates that the protein expression profiles of PM221 and ATCC12228 are very similar whereas RP62A differs extensively from these two strains.

The protein spots that showed statistically significant differences in relative abundances were examined. A total of 119 protein spots displayed at least 1.3-fold change ( $p < 0.05$ ) in at least one of the conditions tested (strain and/or growth stage), and these spots were identified. They included a number of regulators and potential virulence factors. RP62A was found to produce more virulence associated proteins than PM221 and ATCC12228. For example, CodY which is a regulator contributing to virulence and stationary phase adaptation (Sonenshein 2005, Pohl, Francois et al. 2009), was down-regulated in the less virulent strains (PM221 and ATCC12228) in the stationary phase, while its expression was unaffected in both growth phases in RP62A. In addition, the ribosome-associated chaperone, the trigger factor TF, that has been shown to regulate major virulence factors in Gram-positive bacteria (Wu, Zhao et al. 2011), was constantly produced by RP62A, whereas in the other two strains the relative abundance of this factor was clearly reduced in stationary phase cells. With respect to the major virulence factors, more of the  $\alpha$ - and  $\beta$ -subunits of urease (Ure) (Burne, Chen 2000) were produced in both human strains (ATCC12228 and RP62A) than in PM221. On the other hand, catalase which is an enzyme helping the bacterium to combat oxidative stress, in niche competition on nasal mucosa and in intracellular survival of SA (Park, Nizet et al. 2008, Das, Bishayi 2009), was more extensively produced in both growth phases in ATCC12228 compared to PM221 and RP62A, and this could be confirmed in an *in vitro* catalase activity assay.

### **4.4 PM221 and ATCC12228 coordinate TCA cycle and SCV formation in similar ways (I)**

In addition to the differences in the expression of virulence-related proteins, there were also differences in the expression of proteins that are involved in the tricarboxylic acid (TCA) cycle and in bacterial survival during late stationary phases of growth. These proteins included the molecular chaperone ClpC ATPase, pyruvate oxidase CidC and catabolite control protein A (CcpA) (Patton, Rice et al. 2005, Chatterjee, Schmitt et al. 2009, Sadykov, Hartmann et al. 2011), which were less abundant in the stationary phase cells of PM221 and ATCC12228 in comparison with RP62A in the same growth phase. In addition, stationary phase PM221 and ATCC12228 cells exhibited a reduced abundance of proteins associated with nucleotide/nucleoside metabolism, e.g. thymidylate synthesis. Since the formation of small colony variants (SCVs) that survive extremely well in the stationary phase, is associated with reduced TCA cycle activity and deficiencies in thymidylate synthesis (Chatterjee, Herrmann et al. 2007, Chatterjee, Kriegeskorte et al. 2008), it was investigated whether these differences in protein productions would be reflected in colony formation. The serially diluted cell suspensions of all three strains

were cultured on solid media for different time periods. While the RP62A cells continued to form uniformly sized large colonies throughout the observation period, PM221 and ATCC12228 started to produce colonies with different sizes already after 24h on the solid media. When the culturing continued for a longer period (36h), the proportion of small-sized colonies increased in PM221 and ATCC12228 platings. With regard to viability, the PM221 cells started to undergo lysis at 48h and ATCC12228 after 60h while RP62A cells were still viable after that time point.

Previously, PM221 has been shown to induce persistent clinical mastitis (Simojoki, Salomäki et al. 2011). As the genome- and proteome-level findings revealed a high similarity between PM221 and ATCC12228, the infectious potential of ATCC12228 was tested in an experimental infection (I). The results suggest that ATCC12228 is able to evoke a persistent mastitis in cows, but the course of the disease and the inflammatory reaction in the udder would be milder as compared to the IMI caused by PM221. For example, ATCC12228 infection did not cause any systemic clinical signs such as fever, and there were no changes in the appearance of milk.

#### **4.5 PM221 is able to form a biofilm and exhibits higher lipolytic activity than ATCC12228 (II)**

When the proteome catalogs of PM221 and ATCC12228 (II) were compared, several differences emerged among the surface-bound/secreted proteins. PM221 produced more identifiable proteins involved in biofilm formation, drug resistance, adhesion and immune evasion, whereas ATCC12228 was more efficient in synthesizing a 67 kDa myosin cross-reactive antigen, heavy-metal resistance proteins, and serine- and cysteine-type proteases SspA and SspB, each associated with virulence. As the proteins that PM221 produced in higher amounts included Aap, Aae, SdrE and FbpA, which are known to promote protein and/or DNA-mediated biofilm growth (Rohde, Burdelski et al. 2005, Liduma, Tracevska et al. 2012, Heilmann, Thumm et al. 2003, Sharp, Echague et al. 2012, Merino, Toledo-Arana et al. 2009, Sugimoto, Iwamoto et al. 2013, Yan, Zhang et al. 2014), the protein- and DNA-mediated biofilm formation was tested with both strains. As staphylococci are encountering changing environments during infections, the ability of *SE* strains to grow a biofilm was initially tested in several conditions (unpublished data). These preliminary tests revealed that the biofilm forming activity of PM221 was maximal with low levels of CO<sub>2</sub>, and therefore a low-CO<sub>2</sub> condition was chosen for the adherent growth test. The test revealed that PM221 was capable of adherent growth on a polystyrene support, even though PM221 does not have the *ica* operon genes encoding PIA/PNAG, that often mediates biofilm formation (I).

Comparison of all identified proteins from PM221 and ATCC12228 (II) suggested that PM221 was more efficient in producing lipases. A tributyrin cleavage test was performed to determine whether this activity could be detected *in vitro*. In this test, PM221 was slightly more efficient in cleaving tributyrin, a natural component of milk fat, suggesting that the bovine strain could benefit from this activity when living in milk. On the other hand, ATCC12228 produced more identifiable Geh-type lipases known to contribute to colonization and persistence on skin, the normal niche for this strain (Longshaw, Farrell et al. 2000, Bowden, Visai et al. 2002). In addition, ATCC12228 was more efficient in producing 67 kDa myosin cross-reactive antigen, heavy-metal resistance proteins, and serine- and cysteine-type proteases SspA and SspB, which have been associated with *SE* virulence.

#### **4.6 Trypsin shaving releases more surfacome proteins from ATCC12228 than from PM221 (I)**

In a previous experimental infection study (Simojoki, Salomäki et al. 2011), both PM221 and ATCC12228 were found to be able to cause IMI in cows, but the clinical outcome of the ATCC12228 infection was milder than that caused by PM221. It was decided to examine which cell-surface proteins could contribute to this phenomenon by elucidating the cell-surface proteins of these two strains (I). The cells were grown in two differing conditions, aerobic (A) and microaerophilic (M), mimicking environments with high- and low-levels of oxygen as encountered during an infection. The cell-surface proteins were enzymatically released from the cell surface with trypsin, and analyzed with LC-MS/MS. In both conditions, ATCC12228 produced more identifiable proteins than PM221. The proteins identified exclusively in or with markedly higher identification scores in PM221 included accumulation associated protein Aap, immunodominant antigen B (IsaB) and adhesin SdrE, all promoting bacterial adhesion and immunomodulatory functions (Macintosh, Brittan et al. 2009, Mackey-Lawrence, Potter et al. 2009, Sharp, Echague et al. 2012, Yan, Zhang et al. 2014), whereas proteins more or specifically produced by ATCC12228 included such virulence-associated proteins as PSM $\delta$ , autolysins (AtlE, Aae), and penicillin- and iron-binding proteins. ATCC12228 also seemed to produce more moonlighting proteins with adhesive and immunomodulatory functions, such as elongation factors EfTu, EfTs and EfG, enolase and chaperones (Kainulainen, Korhonen 2014).

#### **4.7 RP62A has the largest exoproteome (III)**

The exoproteomes were studied by analyzing the culture supernatants of the three strains with GeLC-MS/MS (III). The identified exoproteins (PM221: 451, ATCC12228: 395, RP62A: 518) were similarly distributed into clusters of orthologous groups (COG) (Tatusov, Natale et al. 2001) in all strains. The largest COG categories were translation, ribosomal structure and biogenesis (COG, J), followed by energy production and conversion (COG, C) and then carbohydrate, amino acid and nucleotide transport and metabolism (COG, G/E/F). Despite the overall similarities, there were more identified proteins associated with cell wall/membrane/envelope biogenesis (COG, M) and post-translational modification, protein turnover and chaperones (COG, O) in PM221 and RP62A than in ATCC12228.

All proteins were classified either as cytoplasmic or extracellular proteins on the basis of several prediction tools (PSORTb, SignalP, SecretomeP, TMHMM, PRED-LIPO, and ScanProsite) and manual inspection. The majority of the identified proteins isolated from growth media were predicted to be cytoplasmic, comprising ~80% of all identifications (371, 313 and 415 proteins in PM221, ATCC12228 and RP62A, respectively). The virulent RP62A strain produced the highest number of identifiable proteins that are primarily considered to be surface-attached and extracellular proteins (103; PM221, 80; ATCC12228, 82 proteins). There were many proteins identified with the classical N-terminal signal sequence for secretion i.e. 50 in PM221, 62 in ATCC12228 and 63 in RP62A. Also integral membrane proteins with transmembrane domains, lipoproteins with the characteristic lipobox motif, as well as proteins with the LPXTG motif mediating covalent anchoring to the cell wall, and the LysM motif which is known to be involved in noncovalent association to cell wall peptidoglycan were identified in the exoproteomes. (Scott, Barnett 2006)

#### 4.8 The exoproteomes of PM221 and RP62A are more similar to each other than to ATCC12228 (III)

There were over 330 strain-shared identifications among the identified exoproteins. Additionally, there were 48 (PM221), 22 (ATCC12228) and 98 (RP62A) proteins that were identified from only one of the strains. These included evolutionary unique proteins and proteins that were strain-shared but produced at an identifiable level only in one strain. In PM221, these specifically identified proteins included proteins related to virulence such as glutamyl aminopeptidase, the ABC transporter OpuCA and methicillin-resistance associated proteins LytH, HmrA and FmhA. RP62A was the most efficient strain in exporting immunomodulatory antigens (IsaB), PSMα, adhesive proteins (SesE, SesG), and different lipolytic, proteolytic and hydrolytic (Ure) enzymes. An *in vitro* urease activity test was conducted to determine how the identification of urease (Ure), an important virulence factor, would be reflected in the urease activity of the culture media. This assay confirmed that the RP62A supernatant had the highest urease activity compared to the less virulent bovine and human strains, although ATCC12228 also exhibited a moderate level of urease activity.

The comparison of exoproteomes revealed that the exoproteomes of PM221 and RP62A were more alike to each other than to ATCC12228. There were also substantial differences in the identification scores of the proteins that were identified in all three strains. For example, β-lactamase (Bla), an enzyme that is involved in antibiotic resistance, was identified in all strains, but with markedly higher scores in PM221 and RP62A as compared to ATCC12228. This indicated that the level of β-lactamase was higher in the supernatants of PM221 and RP62A, and this was verified with an *in vitro* lactamase activity test. This test confirmed that PM221 and RP62A supernatants had higher lactamase activity than ATCC12228, and it was concluded that Bla was being more efficiently exported by PM221 and RP62A under these test conditions.

The comparison between the exoproteome identifications (III) and the total proteome identifications (II) revealed that a large group of proteins were produced by both PM221 and ATCC12228 (II), but secreted out of the cell only by PM221 (III). There was also a large group of proteins produced by both RP62A (III) and ATCC12228 (II), but which were secreted out of the cell by only RP62A. These two groups were strongly overlapping indicating that PM221 and RP62A export similar proteins into the extracellular milieu.

#### 4.9 SE is likely to use non-classical secretion routes in protein export (III)

Several of the predicted cytoplasmic proteins that were identified from the exoproteomes have been previously reported to be moonlighting proteins (Table 5) (Kainulainen, Korhonen 2014). For example, the glycolytic enzymes fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been postulated to function as intercellular signal molecules when they are attached outside the bacteria (Henderson, Martin 2013).



**Table 5.** List of putative moonlighting proteins identified in PM221 exoproteome.

Gene ID	Protein
SEB00228	DNA-directed RNA polymerase beta subunit
SEB00312	Staphylococcal accessory regulator A
SEB00390	1-phosphofructokinase
SEB00430	Ribonucleotide reductase of class Ib (aerobic), alpha subunit
SEB00431	Ribonucleotide reductase of class Ib (aerobic), beta subunit
SEB00473	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase
SEB00474	Phosphoglycerate kinase
SEB00475	Triosephosphate isomerase
SEB00476	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
SEB00477	Enolase
SEB00638	Glucose-6-phosphate isomerase
SEB00767	Pyruvate dehydrogenase E1 component alpha subunit
SEB00768	Pyruvate dehydrogenase E1 component beta subunit
SEB00769	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
SEB00770	Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex
SEB00965	Glutamine synthetase type I
SEB01021	Low-specificity L-threonine aldolase
SEB01260	Manganese superoxide dismutase; Superoxide dismutase [Fe]
SEB01288	Chaperone protein DnaK
SEB01397	Pyruvate kinase
SEB01398	6-phosphofructokinase
SEB01473	Transaldolase
SEB01643	Heat shock protein 60 family chaperone GroEL
SEB01734	Fructose-bisphosphate aldolase class II
SEB01747	Deoxyribose-phosphate aldolase
SEB02002	Phosphoglycerate mutase
SEB02159	Fructose-bisphosphate aldolase class I
SEB02347	Inosine-5'-monophosphate dehydrogenase

In addition to the moonlighting proteins displayed at the cell-surface, another type of non-classical protein export of the cytoplasmic proteins could occur via membrane vesicles (MVs). This MV hypothesis was tested by filtering the culture supernatant through a membrane with 100 kDa cutoff and comparing the protein profiles of the control supernatant, retentate and flow-through fractions using SDS-PAGE and SYPRO-Orange staining. The visualization revealed that the retentate contained proteins with a broad range of molecular masses, including low-molecular mass (<100 kDa) proteins that would have been in the flow-through fraction if they had not been associated with larger structures such as MVs. Additionally, there was no overall enrichment of low-molecular-weight proteins in the flow-through fraction, which also implies that these small cytoplasmic proteins were being exported in high-molecular-weight structures.

The major results of the three studies (I, II, II) are summarized in the Table 6.

**Table 6.** Summary of the major results. The + and – represent the relative results and are not quantitative. N/A = not tested. The last row is a summary statement based on the overall estimation of the results.

	PM221	ATCC12228	RP62A
Genome analysis (I)	resembles ATCC12228	resembles PM221	more distant to PM221 and ATCC12228
Proteome dynamics (I)	clusters with ATCC12228	clusters with PM221	forms a separate cluster
No. of surfacome proteins (moonlighting) (I)	+	++	
Total proteome (II)	similar to ATCC12228	similar to PM221	
Exoproteome (III)	similar to RP62A	differs from PM221 and RP62A	similar to PM221
Formation of SCVs (I)	+	+	-
Catalase activity (I)	+	+++	++
Biofilm forming (II)	+	-	++
Tributylin activity (II)	++	+	
Beta-lactamase activity (III)	+++	++	+++
Urease activity (III)	+	++	+++
Non-classical secretion (III)	++	+	++
Experimental infection (I)	++	+	
Virulence capacity (summary)	+	-	++



## 5 DISCUSSION

The present thesis is composed of three published studies aiming to uncover the infectious and adaptive potentials of the *SE* species. The *SE* strains selected were isolated from bovine (IMI-associated strain PM221) and human (the commensal-type and low-virulent ATCC12228 strain and the sepsis-associated strain RP62A) hosts. First, the genome sequence of the PM221 was defined and compared with the published and unpublished genome sequences of other *SE* and staphylococci (unpublished data). Then proteomic approaches (2D DIGE and GeLC-MS/MS) were used on the different cellular compartments to reveal the mechanisms contributing to virulence and adaptation as well as to complement the genome-level findings.

The genome-level analyses indicated that, while PM221 is very similar with the low-infectious ATCC12228 strain, this bovine strain shares also some genetic factors (e.g. ACME element, genes for Aap, haemolysins, proteases and lipases) which are characteristics of the virulent *SE* strains (I). The results are in line with previous reports revealing that IMI-causing *SE* strains share the same genotype with human isolates and also produce the same virulence factors (Thorberg, Kuhn et al. 2006, Sawant, Gillespie et al. 2009, Jaglic, Michu et al. 2010, Piessens, Van Coillie et al. 2011). This supports the hypothesis that humans could be an important source of the bovine mastitis causing *SE* strains. The PM221 genome was also found to harbor the *fdh* (formate dehydrogenase) gene that is considered as a marker gene which can distinguish between non-pathogenic/commensal and pathogenic-type *SE* strains (Conlan, Mijares et al. 2012). These findings indicate that this marker gene may not be applicable for analyzing bovine-associated *SE* strains. Other distinctive features of the PM221 genome were the higher numbers of gene paralogs and prophage-type elements, which may have provided the bovine strain with better ability to survive and adapt within its bovine host. Another strain-specific feature was the *aap* gene coding for the accumulation associated protein (Rohde, Burdelski et al. 2005). This gene was carried by one of the plasmids in PM221, which could contribute to the adaptation- and biofilm forming-capacity of PM221 as well as facilitate the transfer of this genetic feature to other strains.

The 2D-DIGE analysis (I) that was used to confirm the genome-level findings demonstrated that the protein expression patterns of PM221 during the logarithmic and stationary phases of growth are more similar to those of the commensal strain ATCC12228. The 2DE-based proteomics suffers from several limitations such as restricted dynamic range, difficulties in identification of hydrophobic proteins as well as problems in resolving low abundance proteins with extreme pI and molecular weights (Gilmore, Washburn 2010). Therefore, GeLC-MS/MS was used in surfacome (I), total proteome (II) and exoproteome (III) cataloging to maximize the number of protein identifications. This approach has proved to be effective in generating high-quality and near-to-complete protein catalogs of SA and other bacterial species (Becher, Hempel et al. 2009, Savijoki, Lietzen et al. 2011, Wolff, Hahne et al. 2008, Hempel, Pane-Farre et al. 2010, Beganovic, Guillot et al. 2010) as well as in discovering strain-specific marker proteins of closely related bacteria (Savijoki, Lietzen et al. 2011). The methods used were complementary. The 2D DIGE experiment (I) shed light on the proteome dynamics in different growth phases and highlighted the similarities between the total proteomes of PM221 and ATCC12228. The use of GeLC-MS/MS (I, II, III), in turn, substantially increased the number of identifications. It also elucidated the more virulent nature of PM221 and RP62A compared to ATCC12228, and indicated that PM221 and RP62A are probably able to exploit non-classical secretion mechanisms more efficiently than

ATCC12228 in order to establish infections. Although the surfacome shaving experiment did not result in a large number of new identifications, this analysis indicated the surface-associated location of several proteins and showed that the commensal strain ATCC12228 presents more moonlighting proteins on the cell surface than the mastitis causing strain PM221. Additional comparative surfacome shaving analyses of several strains will still be needed before one could postulate that active moonlighting could be a general attribute of a commensal strain.

A more detailed comparison of the protein expression patterns between the selected three strains further indicated that the bovine (PM221) and the commensal-type (ATCC12228) strains used less aggressive strategies to increase viability and persistence (e.g., down-regulation of the TCA cycle activity, increased formation of SCVs). The higher virulence capacity of RP62A was probably promoted by the increased expression of virulence and adaptation factors (coordinated by TF and CodY) and/or by producing persister cells in response to reactive oxygen species formed during the later stages of growth. Some SA strains have been shown to down-regulate the TCA cycle activity and to enhance the formation of SCVs as ways to improve persistence (Chatterjee, Herrmann et al. 2007, Chatterjee, Kriegeskorte et al. 2008, Chatterjee, Maisonneuve et al. 2011), and the SCV phenotype has recently been described in SE strains associated with prosthetic joint infections (Maduka-Ezeh, Greenwood-Quaintance et al. 2012, Bogut, Niedzwiadek et al. 2014). Since the proteomic analyses indicated that, in addition to PM221, also the ATCC12228 strain may be able to induce IMI, this hypothesis was tested in vivo using a bovine experimental infection model (I). The infection test demonstrated that the ATCC12228 human strain was able to cause mastitis, albeit with milder clinical symptoms than those encountered with PM221. This ability to colonize and infect the bovine host might be partly explained by the large number of the adhesive moonlighting proteins identified from the cell-surface of ATCC12228. Also specific virulence factors (e.g. PSM $\delta$ , AtlE, Aae, SdrG, SspA/B) were found to be expressed by ATCC12228 despite its low-virulent phenotype (I, II). The more detailed comparison of the total proteome catalogs (II) and the identified surfacomes of PM221 and ATCC12228 revealed also differences with likely roles in virulence and/or adhesion. These results suggested that PM221 would be more efficient in producing certain proteins involved in adherence, biofilm formation (e.g. Aap), signal transduction, house-keeping functions, lipolysis (tributyryl cleaving activity) and/or immune evasion. The more pronounced production of lipolytic enzymes by PM221 was reflected in the tributyrin cleavage test which revealed that PM221 was more active than ATCC12228 in cleaving this natural component of cow milk fat. In addition, the higher number of biofilm-associated proteins from PM221 was reflected in the adherent growth test demonstrating that the bovine strain was able to grow as a biofilm in vitro. Both of these features could help the PM221 strain to adapt to the bovine host and to environments containing milk. On the other hand, the higher urease activity of the human strains may reflect the different ecological niches of these bacteria; ATCC12228 and RP62A live on human skin, on mucous membranes and on indwelling medical devices, which all are environments where the bacteria can come into contact with urea as part of body fluids (Burne, Chen 2000).

The total-proteome and surfacome cataloging were next complemented with exoproteome profiling to identify all secreted virulence- and adaptation-associated factors (III). These analyses revealed that RP62A possessed the largest exoproteome with the highest number of virulence-related proteins. Both the RP62A and PM221 were more efficient in secreting  $\beta$ -lactamase, suggesting that these strains may have evolved this ability to deal with the  $\beta$ -lactam group of antibiotics, which they are likely to face during an infection. A notable finding was that PM221

and RP62A released a similar set of intracellular proteins into the surrounding environment. The same proteins were found to be synthesized by ATCC12228, but the present proteomic results indicated that these proteins are intracellular and/or are displayed at the cell-surface (I, II). These findings suggest that PM221 and RP62A export these cytoplasmic proteins out of the cell, which may be one of the bacterium's strategies for enhancing virulence and adaptation.

In numerous studies, large amounts of proteins that are theoretically predicted to be localized in the cytoplasm have been identified on the surface or outside of the cell (Tjalsma, Lambooy et al. 2008, Dreisbach, Hempel et al. 2010, Solis, Larsen et al. 2010, Ythier, Resch et al. 2012). This was also the case in this study (III). It has been suggested that the concept of "subcellular location" should be revised so that it should be seen rather as dynamic trafficking between different subcellular locations (Olaya-Abril, Jimenez-Munguia et al. 2013). Thus, instead of being described as cytosolic proteins found in the secretome or surfacome, these proteins could be termed "proteins for which algorithms of subcellular location predict to be in the cytoplasm, because lacking exporting/retention signals", in other words as "non-classically secreted proteins" (Olaya-Abril, Jimenez-Munguia et al. 2013).

How do the cytoplasmic proteins end up in the exoproteomes of the bovine and human *SE* strains? Three hypotheses offer plausible explanations, although these are not mutually exclusive.

First, they might be in this location merely due to cell lysis (Tjalsma, Lambooy et al. 2008). In the present study, all three strains grew similarly in the growth media used, suggesting that there should not be any major differences in cell lysis susceptibilities between the strains.

Second, they might be moonlighting proteins that have been exported to the cell-surface via some non-classical secretion route and then released during centrifugation or by some protease-assisted mechanism e.g. as described for *B. subtilis* (Chhatwal 2002, Henderson, Martin 2011, Krishnappa, Dreisbach et al. 2013). The moonlighting proteins are secreted especially during the stationary growth phase, and at least for some of them protein domain structure modifications can contribute to this phenomenon (Yang, Ewis et al. 2011). Certain stress conditions favor the secretion of moonlighting proteins, for example high bile salt and glucose concentrations, elevated temperature and starvation, i.e. conditions that are thought to be part of the environment in which the bacteria live and that can affect the cell wall as well as membrane organization and permeability (Candela, Centanni et al. 2010, Saad, Urdaci et al. 2009, Wang, Xia et al. 2013, Kainulainen, Loimaranta et al. 2012). After being exported, the secreted moonlighting proteins become attached to the cell surface. In gram-positive bacteria, this might be promoted by the negatively charged lipoteichoic acids of the cell envelope, and by the pH of the environment that can affect the net charge of moonlighting proteins (Kainulainen, Loimaranta et al. 2012, Antikainen, Kuparinen et al. 2007b). For example, ribosomal proteins are often found on the surface of bacteria – also on the surface of Gram-positive bacteria – as they have a particularly high affinity for the negatively charged cell wall (DebRoy, Dao et al. 2006, Severin, Nickbarg et al. 2007, Tjalsma, Lambooy et al. 2008, Pocsfalvi, Cacace et al. 2008, Resch, Leicht et al. 2006, Planchon, Chambon et al. 2007, Ruiz, Coute et al. 2009). In fact, they are so often found on the bacterial cell surfaces that they are considered as new anchorless surface proteins (DebRoy, Dao et al. 2006), and some of them have been demonstrated to be immunogenic in SA strains that cause human and bovine infections (Sinha, Kosalai et al. 2005, Geng, Zhu et al. 2008, Nho, Hikima et al. 2011). Also in the present study, over 45 ribosomal proteins were identified in the exoproteomes of all three strains. The exoproteome samples were collected from the late-logarithmic growth phase when the cells were already encountering environmental stresses. These stresses are known to increase the release of moonlighting proteins in several bacteria

(Eichenbaum, Green et al. 1996, Ruiz, Coute et al. 2009, Kainulainen, Loimaranta et al. 2012), and this could serve as a partial explanation for the high number of identified ribosomal and other moonlighting proteins.

Another well-known moonlighting protein, the glycolytic enzyme enolase, was identified in the growth media of all three strains and after surfacome shaving from both PM221 and ATCC12228 strains and in both growth conditions. The moonlighting, surface-associated enolase can act as a receptor for plasminogen leading to the proteolytic activity of plasminogen (Bhattacharya, Ploplis et al. 2012). This activity causes host matrix degradation and damage to connective tissue, and it has been observed to be present in several pathogenic bacteria (Nordstrand, Shamaei-Tousi et al. 2001, Sun, Ringdahl et al. 2004, Jonsson, Guo et al. 2004, Bhattacharya, Ploplis et al. 2012). There are also reports that enolase can protect the bacterium from complement-mediated killing (Agarwal, Hammerschmidt et al. 2012). Other identified adhesive and/or immunostimulatory enzymes observed in the exoproteomes included DnaK, GAPDH and Eftu, which were also found to be present in the surfacomes (I) of PM221 and ATCC12228. The moonlighting chaperone protein DnaK has been claimed to stimulate monocyte chemokine synthesis while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has inhibited the  $H_2O_2$  synthesis of human neutrophils (Wang, Kelly et al. 2001, Terao, Yamaguchi et al. 2006). Enolase, GAPDH and Eftu are also known to be antigenic (Ling, Feldman et al. 2004, Pancholi, Fischetti 1998, Granato, Bergonzelli et al. 2004).

A third possible explanation for the extracellular location could be the export of those proteins via their incorporation inside MVs. Previous studies have demonstrated that SA MVs have distinct protein patterns and contain a high number of extracellular proteins with the protein and fatty acid compositions of MV-membrane differing from those on the plasma membrane (Kuehn, Kesty 2005, McBroom, Johnson et al. 2006, Rivera, Cordero et al. 2010). In the present study, the enrichment of the MV-fraction from culture supernatants (III) indicated that the small (<100 kDa) proteins had become embedded in larger structures, evidence that also SE – as SA – is able to use MVs as a way to secrete proteins in order to ensure full virulence. These observations suggest that the MV-embedded proteins contain some specific sorting mechanism, and that the vesicles are not merely originating from dying cells, bacterial lysis or envelope instability. The MVs provide the bacteria with flexibility in responding to the environmental changes and challenges, because the release of MVs is partly modulated by the ambient conditions such as availability of nutrients and the presence of antibiotics (Deatherage, Cookson 2012). They have also a role in pathogenicity; for example SA has been shown to be able to deliver cytotoxic effector molecules into host cells via MVs both in vitro and in vivo (Gurung, Moon et al. 2011, Thay, Wai et al. 2013). Taken together, the exoproteome results (III) revealed that SE can use different non-classical secretion mechanisms – moonlighting and/or MVs – to enhance its adaptation and virulence.

## 6 CONCLUSIONS AND FUTURE PERSPECTIVES

This study is the first large-scale proteome analysis and the first exoproteome analysis of the *SE* species. The differences and similarities between the bovine (PM221) and human (ATCC12228, RP62A) strains were elucidated, pinpointing potential strain-specific factors in virulence and adaptation, and confirming the genome-level protein predictions. At the genome and total proteome levels, the bovine strain PM221 was found to be more similar to the low-virulent and commensal-type strain ATCC12228 than to the sepsis-associated strain RP62A. Both PM221 and ATCC12228 were able to induce persistent IMI in an experimental bovine infection model, although the clinical outcome with the human strain was milder than that encountered with the bovine strain. These findings strengthen the hypothesis that humans could indeed be the source of bovine mastitis causing *SE* strains.

In attempt to explain their full infectious potential, the exoproteomes of each three *SE* strains were compared. This comparison indicated that the exoproteome of PM221 resembled more that of RP62A than the exoproteome of ATCC12228. This finding together with the results demonstrating that PM221 and RP62A are more efficient in non-classical protein secretion, may explain the higher virulence capacity of PM221 than that of ATCC12228. These studies also indicated that PM221 uses less aggressive strategies than RP62A when establishing an infection *in vivo*.

Although more phenotypic, immunological and mutational tests are needed to confirm the biological and medical relevance of these results, they do represent the first major large-scale proteomic characterization of this important pathogen, and identify potential candidates for future studies. Hopefully these results can serve as a stepping stone in the search for preventive and therapeutic solutions against staphylococcal infections.

In future studies, the opportunistic nature of *SE* should be addressed; how the transition from commensalism to pathogenicity is reflected in the proteome. To accomplish this, first the genomes of several *SE* strains of both bovine and human origin should be sequenced. Then, the predicted surfacome and exoproteome proteins should be compared in order to find specific markers for commensalism and pathogenicity. After this genome-level analysis, the effect of different growth conditions on the expressed proteomes – especially surfacomes and exoproteomes – should be examined with proteomic approaches. These different growth conditions could include cultivation in growth media containing different host-derived components, growth in bovine and human serum, growth in the presence of host cells, and/or cultivation with a variety of known stress factors such as changes in pH- and CO<sub>2</sub>-levels.

Since this present study indicates that *SE* produces MVs, the biological relevance, protein composition, antigenicity, role in virulence and effects on the target cells of these secreted MVs should also be addressed. To accomplish this, the MVs of several *SE* strains should be visualized by electron microscopy, isolated, and the proteins embedded in MVs will need to be identified. The major findings would then be confirmed with phenotypic tests, and the antigenicity of the identified proteins should be evaluated with immunological methods. The adhesion and possible invasion to the target cells could, in turn, be studied with both human and bovine epithelial cell models.



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